Chronic insulin treatment suppresses PTP1B function, induces increased PDGF signaling, and amplifies neointima formation in the balloon-injured rat artery

Qinghua Pu, Yingzi Chang, Chunxiang Zhang, Yi Cai, and Aviv Hassid
Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee
Submitted 9 April 2008; accepted in final form 3 November 2008

Pu Q, Chang Y, Zhang C, Cai Y, Hassid A. Chronic insulin treatment suppresses PTP1B function, induces increased PDGF signaling, and amplifies neointima formation in the balloon-injured rat artery. Am J Physiol Heart Circ Physiol 296: H132–H139, 2009. First published November 14, 2008; doi:10.1152/ajpheart.00370.2008.—We tested the hypothesis that hyperinsulinemia induces the suppression of protein tyrosine phosphatase 1B (PTP1B) function, leading to enhanced PDGF receptor (PDGFR) signaling and neointimal hyperplasia. Rats were implanted with insulin-releasing pellets or sham pellets. Blood glucose levels, insulin levels, food and water intake, body weights, and blood pressures were measured. Neointimal hyperplasia was assessed by computerized morphometry 14 days after carotid balloon injury. PTP1B protein expression in injured arteries was determined via Western blot analysis, whereas PTP1B activity was determined via an immunophosphatase assay. Serum insulin levels were two- to threefold greater in hyperinsulinemic rats, whereas systolic blood pressures, food and water intake, serum triglyceride levels, plasma cortisol levels, and urinary catecholamine levels were not affected. Fourteen days after injury, neointima-to-media area ratios were 0.89 ± 0.23 and 1.35 ± 0.22 in control and hyperinsulinemic rats, respectively (P < 0.01). PTP1B protein levels and total PTP1B activity in injured carotid arteries from the insulin-treated group were significantly decreased 7 or 14 days after injury, whereas PTP1B specific activity was decreased only 14 days after injury. These findings were associated with decreased PTP1B mRNA levels and increased PDGFR tyrosyl phosphorylation in insulin-treated rats. These observations support the hypothesis that hyperinsulinemia induces the suppression of PTP1B function, leading to enhanced PDGFR signaling and neointimal hyperplasia.

restenosis; reverse transcription-polymerase chain reaction; vascular injury; protein tyrosine phosphatase 1B; platelet-derived growth factor

HYPERINSULINEMIA is a key feature of metabolic syndrome and type 2 diabetes (18). Moreover, plasma insulin levels in the population of the United States appear to have increased by ~35% during the last decade (32). It is now well accepted that metabolic syndrome and type 2 diabetes are strongly associated with adverse cardiovascular events in humans, although the underlying reasons for these associations have not been clarified (3, 12, 44, 49, 55). Several recent studies have reported that hyperinsulinemia is associated with vascular remodeling and enhanced neointima formation in models of experimental vascular injury as well as in humans (20, 28, 37, 39, 55). Moreover, hyperinsulinemia, but not hyperglycemia, has been reported to induce enhanced neointima formation in models of rat vascular injury (11, 28).

The migration of medial vascular smooth muscle cells to the intima and the subsequent cell proliferation are thought to be necessary for the vascular remodeling that occurs after injury or in atherosclerosis. Acute treatment with insulin has been reported to attenuate the motility and proliferation of cultured vascular smooth muscle cells (11), whereas chronic insulin treatment has the capacity to enhance the motility and proliferation of cultured smooth muscle cells (23, 53). A clinical meta-analysis study (40) has reported a correlation between hyperinsulinemia and cardiovascular disease, and another study (36) found that the cumulative dose of insulin treatment in type 1 diabetics is associated with increased neointimal thickening, suggesting that insulin may enhance vascular smooth muscle cell motility in humans.

Protein tyrosine phosphatases (PTPs) are thought to play an important role as counterregulatory agents that attenuate or terminate signaling induced by the activation of receptor tyrosine kinases. PTP1B is a ubiquitously expressed nonreceptor phosphatase targeted to several intracellular domains, including the endoplasmic reticulum and focal adhesions (2, 21). PTP1B has been most prominently linked with the regulation of insulin or IGF-I signaling in vitro and in vivo (1, 5, 15, 30, 57). We (43) have reported that PTP1B attenuates the motility of dedifferentiated cultured smooth muscle cells isolated from newborn rat induced by acute treatment with insulin via dephosphorylation of tyrosyl in insulin receptors. In another study (8), we reported that PDGF and FGF, but not IGF-I, significantly increased levels of PTP1B protein in differentiated cultured rat aortic smooth muscle cells from adult rats. We (7) have also found that PTP1B plays an important role in attenuating PDGF-induced cell motility in culture and neointima formation in vascular injury. These findings, taken together, indicate that upregulation of PTP1B is part of a negative feedback mechanism that modulates vascular remodeling. Recently, we (56) have reported that chronic but not acute insulin treatment enhances the motogenic effect of PDGF by suppressing PTP1B levels and activity in differentiated cultured rat aortic smooth muscle cells via a mechanism involving enhanced PDGF receptor (PDGFR) phosphorylation.

The aim of the present work was to test the hypothesis that hyperinsulinemia suppresses the upregulation of PTP1B in a rat carotid artery injury model. We report the novel findings that an infusion of insulin into the rat, via an insulin-releasing pellet, increases plasma insulin levels by two- to threefold, without eliciting hypertension or hypertriglyceridemia, and...
that this treatment induces a marked suppression of PTP1B levels and activity and increases PDGFR tyrosyl phosphorylation, together with enhanced neointima formation.

MATERIALS AND METHODS

Materials. Adult male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA). Insulin-releasing pellets (Linplant) and sham (control) pellets were from Linshin Canada (Toronto, ON, Canada). The rat/mouse insulin enzyme-linked immunosorbent assay kit was obtained from Linco Research (St. Charles, MI). Monoclonal antibody against PTP1B was purchased from BD Biosciences (Franklin Lakes, NJ), and polyclonal antibody against PDGFR was from Upstate (Lake Placid, NY). Phosphatase inhibitor cocktail was from Calbiochem (San Diego, CA), protease inhibitor cocktail and a kit for the measurement of triglyceride levels were from Sigma (St. Louis, MO). Protein G-Sepharose beads were from Amersham Pharmacia Biotechnology (Piscataway, NJ). IR3,9 insulin receptor peptide (1142–1153) (pY1142, pY1150) was from BioMol Research Laboratories (Plymouth Meeting, PA). Goat polyclonal antibody against PDGFR-β, rabbit polyclonal against PDGFR-β, and rabbit polyclonal antibody against CDK4 were from Santa Cruz Biotechnology (Santa Cruz, CA), and phospho-specific antibody against PDGFR-β (targeting phospho-Y751) was from Cell Signaling (Boston, MA). The cortisol ELISA kit and urine metanephrine ELISA kit were from IBL (Hamburg, Germany).

Animals and experimental design. Male Wistar rats weighing 180–200 g were housed in a light-controlled environment on an alternating 12:12-h light-dark cycle. Room temperature was kept at 20–23°C, and humidity was at 55–60%. Rats were maintained on standard rat chow, and all rats had free access to food and water except when scheduled for fasting. All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and were performed under aseptic conditions in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23).

After 1 wk of acclimatization and training for habituation to tail-cuff blood pressure recording, rats were randomly allocated to two groups. Hyperinsulinemia was implemented by following a previously published procedure (6). Briefly, rats received a 2-mm segment of a 7-mm blank or sustained-release bovine insulin-releasing pellet (the expected delivery rate of insulin was 0.6 U/day for >40 days as indicated by the manufacturer of the pellet) under short-term isoflurane anesthesia (model 100 vaporizer, Surgivet) and kept in 1.5-ml Eppendorf tubes on ice. Serum was separated by centrifugation at 4°C and stored at −20°C. Serum insulin levels were determined via an ELISA-based direct sandwich technique. Cross-reactivity levels of the assay were as follows: rat 100% and bovine insulin 78%. The sensitivity threshold for this assay was 35 pmol insulin. Rat C-peptide and leptin were not detectable up to 120 nM. For the measurement of insulin, blood samples were collected after an overnight fast on weeks 0, 1, 3, and 5 (n = 12 in the control group and 12 in the insulin group). For the measurement of triglycerides, blood samples were collected after an overnight fast on week 4 (n = 6 in the control group and 6 in the insulin group). Serum triglyceride levels were determined by measurements of glycerol released after an enzymatic hydrolysis reaction (31).

For the measurement of plasma cortisol, fed rat blood samples were collected on weeks 1 and 3 (n = 7 in the control group and 7 in the insulin group). Plasma was prepared by collecting 300 μl of blood into a tube coated with EDTA (final concentration of EDTA: 0.3%) and centrifuging for 20 min at 4°C followed by the collection of supernatant. The cross-reactivity characteristics of this assay were as follows: cortisol 100%, corticosterone 45%, and deoxycorticisol <2%. The sensitivity threshold was 2.5 ng/ml.

Measurement of urinary catecholamine levels. Urinary metanephrine levels were measured as previously described (50). After insulin or control pellet implantation, rats were housed individually in metabolic cages (n = 7 in the control group and 7 in the insulin group) with free access to water and food. After a familiarization period of 24 h, urine samples were collected from animals for a period of 24 h at week 1 and 3 time points (1 wk after the first pellet implantation and 1 wk after the second pellet implantation, respectively) and frozen in liquid nitrogen until analysis. Urine metanephrine levels were measured via ELISA. The cross-reactivity characteristics of this assay were as follows: metanephrine 100%, epinephrine 0.33%, and normetanephrine 0.15%. The threshold sensitivity was 8.6 ng/ml.

Measurement of blood glucose levels. Tail tips of rats were nicked with a needle, a drop of blood was loaded on a glucose strip, and blood glucose levels were measured with a glucose meter (Onetouch Ultra Meter, Johnson & Johnson). Nonfasting and overnight fasting blood glucose levels were measured on weeks 0, 1, 3, and 5.
Measurement of blood pressure. Systolic blood pressures in 12 control and 12 insulin-treated rats were measured by the tail-cuff method (XBP1000, Kent Scientific) as previously described (48).

Measurement of food and water intake. Twelve rats in the control group and twelve rats in the insulin-treated group were kept in individual cages, and food containers and water bottles were weighed before and after a 24-h period during week 5. The differences in weight at the beginnings and ends of 24-h measurement periods were considered to represent food and water intake.

Immunoprecipitation and Western blot analysis. Frozen carotid arteries were pulverized in liquid nitrogen and subjected to lysis in buffer containing 20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM Na3VO4 supplemented with protease inhibitors (containing 0.5 mM PMSF, 0.4 μM aprotinin, 10.5 μM leupeptin, 18 μM bestatin, 7.5 μM peptatin A, and 7 μM E-60). Equivalent amounts of protein (500 μg/sample) estimated by bicinchoninic acid reagent (Pierce) were incubated with 2 μg anti-PDGFR-β antibody overnight at 4°C. Immunocomplexes were precipitated with 40 μl 1:1 slurry protein G-Sepharose after a 1-h incubation at 4°C, boiled in Laemmli sample buffer, and centrifuged. The resulting supernatants were subjected to electrophoresis on SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore). Immunoblot analysis was performed using phospho-PDGFR-β antibody (Y751). Immunocomplexes were visualized using an ECL method (Amersham). After the antibody targeting phosphotyrosyl was stripped off, membranes were immunoblotted with anti-PDGFR-β antibody as described above. Western blot band densities were measured using the public domain NIH Image program.

Arterial PTP1B levels were determined by Western blot analysis via standard protocols. Blots were probed with an antibody directed against PTP1B. Equivalent loading of protein was verified by reprobing with antibody against CDK4 (13).

PTP1B activity assay. Rats were killed 7 or 14 days after injury. Three to four experiments were performed for each experiment. For each experiment, carotid arteries from six insulin-treated rats or six control rats were pooled, ground in liquid nitrogen, and homogenized in 500 μl of ice-cold HEPES lysis buffer of the following composition: 50 mM HEPES, 150 mM NaCl, 2.5 mM EDTA, and 1% Triton X-100 supplemented with protease inhibitor cocktail (0.5 mM PMSF, 0.4 μM aprotinin, 10.5 μM leupeptin, 18 μM bestatin, 7.5 μM peptatin A, and 7 μM E-60) and serine/threonine phosphatase inhibitor cocktail (25 mM β-mercaptoethanol) at 30°C for 1 h with continuous shaking. This tyrosine-phosphorylated peptide has been used previously as a PTP substrate (42). After an incubation, samples were put on ice to terminate the reaction, and 25 μl of supernatant were transferred to a 96-well plate; the total volume was brought to 100 μl with assay buffer. Malachite green solution (20 μl) was added into each well, and samples were gently agitated for 10 min. The free phosphate liberated was determined by reading the absorbance at 650 nm (corresponding to the malachite green-phosphate complex) using a microtiter-plate spectrophotometer. A standard curve was generated for each experiment to quantify results. A portion of the immunoprecipitates was used to determine the levels of PTP1B via Western blot analysis. Enzyme activity results were normalized either to total protein levels (total activity) or to the quantity of PTP1B in the immunoprecipitates (specific activity).

Measurement of PTP1B mRNA levels via semiquantitative real-time PCR. RNA was extracted from 20 mg of tissue (2 vessels pooled per sample) via a kit (Aurum, total RNA sample pack) from Bio-Rad (Hercules, CA). mRNA was reverse transcribed into cDNA with the use of a Transcriptor First Strand cDNA Synthesis kit from Roche (Indianapolis, IN) followed by the performance of real-time PCR for PTP1B using a LC 480 Real-Time PCR instrument (Roche Diagnostics). Results were normalized against cyclophilin or hypoxanthine-guanine phosphoribosyl transferase (HGPR) mRNA, because the expression of these housekeeping genes has been shown to remain stable in a rat carotid artery injury model (16, 29). Real-time PCR was performed using the following probes derived from Universal probe library software (universalprobepluslibrary.com; Roche Applied Science): PTP1B primer, forward 5′-GGAAACAGGTACCGAGATGTCA-3′ and reverse 5′-AGTCATTCTCCTCTGATGCAAAT-3′; cyclophilin primer, forward 5′-ACCTCCACAGGTCACCCTC-3′ and reverse 5′-ACCTCCACAGGTCATCC-3′; and HGPR primer, forward 5′-GTCACGGGGGACAAAG-3′ and reverse 5′-TGCAATT-TTACCAGGTATCAA-3′.

Statistical analysis. Results are expressed as means ± SE and were analyzed using two-way ANOVA followed by Fisher’s least-significant-difference test or Student’s unpaired t-test. P < 0.05 was considered statistically significant.

RESULTS

Infusion of insulin induced modest hyperinsulinemia, but not hypertension, and elevated glucocorticoid, catecholamine, and triglyceride levels. Our overall aim was to determine the effect of hyperinsulinemia on vascular PTP1B levels and activity in a carotid injury model. Some studies (4, 27) have reported an association between experimental hyperinsulinemia and hypertension or hypertriglyceridemia. We used a model of insulin infusion in the rat on the basis of a report (6) indicating that the use of an implantable insulin-impregnated pellet, in two stages, induces modest hyperinsulinemia without eliciting significant hypertension or hypertriglyceridemia. As shown in Fig. 1, after the second insulin pellet implantation, we obtained a two- to threefold increase of insulin levels without significant hypertension (Table 1), hypertriglyceridemia (control: 0.75 ± 0.18 mg/ml and insulin treatment: 0.67 ± 0.06 mg/ml, 2 wk after the second insulin pellet, P > 0.05), or nonfasting hypoglycemia (Table 2), although we did observe significant hypoglycemia in insulin-infused rats after an overnight fast (Table 2).

![Fig. 1. Insulin levels before and after treatment with insulin. Rats were implanted with sham or insulin-releasing pellets at weeks 0 and 2. Data are means ± SE from 12 rats/group. Arrows indicate the administration of the first and second pellet, respectively. *P < 0.05 compared with control by ANOVA followed by Fisher’s test.](http://ajpheart.physiology.org)
Food intake and drinking behavior were also similar for the two treatment categories, although rats treated with insulin manifested a slight but significant weight gain after 5 wk of treatment (Table 3).

On the basis of reports (24, 47, 51) associating experimental hyperinsulinemia with elevated levels of glucocorticoids and catecholamines, we measured plasma levels of cortisol and urinary levels of catecholamines in hyperinsulinemic rats and normoinsulinemic controls and verified that the rats were indeed hyperinsulinemic (data not shown). As shown in Table 4, hyperinsulinemia did not induce significant changes in the levels of either plasma cortisol or urinary metanephrine.

**Treatment with insulin enhanced neointima formation induced by vascular injury.** Several studies (11, 20, 28, 39) have reported that in at least some strains of rats, hyperinsulinemia induces a significant increase of neointima formation. Thus, an aim of our study was to verify this finding in our model. In confirmation of previous results (28), and as shown in Fig. 2, the formation of neointima was increased by ~50% (without a change of medial area) 14 days after the induction of vascular injury. As shown in Table 4, hyperinsulinemia did not induce significant changes in the levels of either plasma cortisol or urinary metanephrine.

**Hyperinsulinemia suppressed the elevation of PTP1B protein levels induced by vascular injury.** We (8) have reported that PTP1B levels are significantly increased in a model of rat carotid injury. On the basis of this and other aforementioned findings, we tested the hypothesis that insulin would suppress the upregulation of PTP1B induced by vascular injury. As shown in Fig. 3, vascular injury elicited a three- to fourfold increase of PTP1B protein levels both 7 and 14 days after vascular injury, confirming our previous findings. Importantly, in insulin-treated rats, the increase of PTP1B levels was significantly attenuated at both time points compared with rats receiving sham treatment.

### Table 1. Systolic blood pressure levels in insulin-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoinsulinemia</td>
<td>121.17±5.17</td>
<td>116.33±5.78</td>
<td>121.08±6.90</td>
<td>124.20±5.23</td>
</tr>
<tr>
<td>Hyperinsulinemia</td>
<td>119.25±6.54</td>
<td>116.67±6.00</td>
<td>123.25±5.42</td>
<td>124.78±3.99</td>
</tr>
<tr>
<td>P value</td>
<td>0.52</td>
<td>0.91</td>
<td>0.46</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Data are means ± SE (in mmHg); n = 20 rats/group. Systolic blood pressure was measured via tail-cuff sphygmomanometry. *P* values were calculated via ANOVA followed by Fisher’s test.

### Table 2. Effect of insulin treatment on blood glucose levels

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>102.0±27.4</td>
<td>96.4±6.4</td>
<td>92.6±6.4</td>
<td>92.6±6.4</td>
</tr>
<tr>
<td>Insulin</td>
<td>105.0±21.4</td>
<td>97.1±6.1</td>
<td>97.1±6.1</td>
<td>97.1±6.1</td>
</tr>
<tr>
<td>Fasted animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83.8±8.1</td>
<td>76.5±7.8</td>
<td>83.0±8.3</td>
<td>84.9±6.7</td>
</tr>
<tr>
<td>Insulin</td>
<td>84.9±7.7</td>
<td>54.3±5.9*</td>
<td>54.4±12.9*</td>
<td>54.4±12.8*</td>
</tr>
</tbody>
</table>

Data are means ± SE (in mg/dl); n = 12 rats/group. Rats were implanted with control pellets or insulin-releasing pellets on weeks 0 and 1. Blood samples were collected on week 1 (1 wk after first pellet implantation) and week 3 (1 wk after second pellet implantation) via puncture of the retroorbital venous plexus. Plasma cortisol levels were determined via an ELISA-based competitive binding technique (IBL, Hamburg, Germany). For the measurement of metanephrine levels, rats were implanted with control pellets or insulin-releasing pellets on weeks 0 and 2. Urinary metanephrine levels were determined via an ELISA-based competitive binding assay (IBL). No differences were statistically significant.

### Table 3. Effect of insulin on food intake, water intake, and weight gain

<table>
<thead>
<tr>
<th></th>
<th>Food Intake, g/day</th>
<th>Water Intake, ml/day</th>
<th>Body Weight Gain, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.1±2.6</td>
<td>46.9±9.6</td>
<td>126.6±15.1</td>
</tr>
<tr>
<td>Insulin</td>
<td>30.1±2.9</td>
<td>48.3±7.1</td>
<td>145.1±15.6*</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 10 in the control group and 12 in the insulin group. Rats were implanted with control pellets or insulin-releasing pellets on weeks 0 and 2. Food and water intake were measured on 1 day during week 5; body weight gained during 5 wk was determined by weighing body weight on weeks 0 and 5. *P* < 0.05 compared with the control group by Student’s *t*-test.

**Hyperinsulinemia attenuated the elevation of PTP1B activity induced by vascular injury.** Our next experiments were performed to determine the effect of hyperinsulinemia on PTP1B activity in the aforementioned model. To this end, we immunoprecipitated PTP1B and performed an immunophosphatase assay as previously described (26). As shown in Table 5, vascular injury elicited a significant increase of PTP1B activity, in parallel with increased PTP1B protein levels. Treatment with insulin induced a marked and significant reduction of total PTP1B activity levels at both 7 and 14 days after injury. Interestingly, PTP1B specific activity was also significantly decreased by insulin treatment at 14 days but not 7 days after injury.

**Hyperinsulinemia suppressed the increase of PTP1B mRNA levels induced by vascular injury.** A previous study (52) has reported that vascular injury induces marked elevation of mRNA levels of several PTPs, including that of PTP1B (52), and, on the basis of this finding, we tested the hypothesis that insulin would suppress the elevation of mRNA in vascular injury. As shown in Table 6, vascular injury increased PTP1B mRNA levels by approximately twofold 7 days after injury, confirming previous findings. Importantly, insulin treatment prevented the increase of mRNA PTP1B, thus providing a mechanism that explains the reduction of PTP1B protein levels.

**Hyperinsulinemia increased the levels of PDGFR-β tyrosyl phosphorylation in injured carotid arteries.** Substantial evidence supports the notion that PDGF plays an important role in the hypertrophic response induced by vascular injury (22). We (8) have reported that PDGF-BB stimulates the phosphorylation of PDGFR-β in cultured smooth muscle cells at concentrations of 10 ng/ml and greater. Substantial evidence supports the notion that PDGF plays an important role in the hypertrophic response induced by vascular injury (22).

### Table 4. Effect of insulin treatment on plasma cortisol and urinary metanephrine levels (ng/ml)

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>26.2±16.0</td>
<td>37.3±13.3</td>
</tr>
<tr>
<td>Insulin</td>
<td>29.7±12.8</td>
<td>36.1±12.1</td>
</tr>
<tr>
<td>Metanephrine</td>
<td>42.3±9.2</td>
<td>39.7±4.7</td>
</tr>
<tr>
<td>Insulin</td>
<td>43.2±2.7</td>
<td>40.0±7.0</td>
</tr>
</tbody>
</table>

Data are means ± SE (in ng/ml); n = 7 rats/group. For the measurement of cortisol levels, rats were implanted with control pellets or insulin-releasing pellets on weeks 0 and 2. Blood samples were taken from the tail veins of nonfasting rats or from rats after an overnight fast. Blood glucose levels were measured via a glucose meter (Onetouch Ultra Meter, Johnson & Johnson). *P* < 0.05 compared with the control group by Student’s *t*-test.

**Discussion**

The results presented here confirm previous findings that insulin treatment significantly reduce the formation of neointima in a model of vascular injury (24, 47, 51). The mechanism that explains the reduction of PTP1B protein levels induced by vascular injury is not yet understood. However, previous reports have shown that hyperinsulinemia decreased PTP1B specific activity in cultured smooth muscle cells (8). In the present study, we have shown that hyperinsulinemia suppressed the elevation of PTP1B mRNA levels induced by vascular injury. Importantly, insulin treatment prevented the increase of mRNA PTP1B, thus providing a mechanism that explains the reduction of PTP1B protein levels.
neointima formation via activation of the βI isoform of PDGFR (14, 17, 22, 54). These findings prompted us to test the hypothesis that the suppression of PTP1B function by treatment with insulin would enhance PDGFR-β phosphotyrosyl levels in injured rat carotid arteries. We observed a modest but insignificant increase of PDGFR-β phosphorylation 7 days after injury; on the other hand, we observed a robust and statistically significant increase of approximately twofold in the phosphotyrosyl levels of PDGFR-β 14 days after injury (Fig. 4).

**DISCUSSION**

It is now well recognized that signaling events downstream of polypeptide growth factor receptors involve the activation of intrinsic receptor tyrosine kinases. It is also well established...
PTP1B/cyclophilin 1 2.11/H11006

Table 6. Effect of insulin treatment on mRNA levels of PTP1B in normal or injured carotid arteries

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Insulin</th>
</tr>
</thead>
</table>
| PTP1B/HGPRT | 1       | 2.05±0.35
|            |         | 1.27±0.26
|            |         | 1.04±0.32* |
| PTP1B/cyclophilin | 1 | 2.11±0.10 |
|            |         | 1.21±0.20 |
|            |         | 1.28±0.20* |

Data are means ± SE (expressed as fold above control); n = 3 experiments with 6 rats/group. Rat were implanted with control pellets or insulin-releasing pellets followed by balloon injury to the right carotid arteries. Both injured and uninjured carotid arteries were removed 7 days after injury, and two arteries from the same treatment category were pooled to generate one sample. mRNA levels for PTP1B were measured via real-time PCR after reverse transcription into cDNA. Two housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and cyclophilin, were used as controls. *P < 0.05 compared with injured control arteries by ANOVA followed by Fisher’s test.

that neointimal expansion induced by vascular injury is causally associated with growth factor receptors that function as tyrosine kinases (14, 17, 22, 33, 54). Recent studies (19, 25, 35, 38) have indicated that steady-state phosphotyrosyl levels generally represent the balance of tyrosine kinase activity opposed by tyrosine phosphatase activity. Although mechanisms related to tyrosine kinases have undergone extensive scrutiny, relatively little is known about the mechanisms related to tyrosine phosphatase function.

PTP1B is a ubiquitously expressed so-called “nonreceptor” phosphatase targeted to several intracellular domains, including membrane-proximal domains, the endoplasmic reticulum, and focal adhesions (2, 25). We (7) have previously reported that treatment of cultured cells with growth factors or the induction of vascular injury in the rat elicits an upregulation of PTP1B and that interference with PTP1B signaling via the use of dominant negative PTP1B enhances neointima formation, thus uncovering a negative feedback pathway involving PTP1B.

Insulin has been reported to amplify neointima formation in experimental models (28, 39). Some, but not all, studies have reported that there is a significant correlation between the levels of insulin and vascular remodeling in humans (44, 55), although the mechanisms explaining this finding are lacking. Studies have also reported that insulin suppresses the activity of PTP1B in various tissues, although evidence to the contrary also exists (10, 34, 45, 46). In a recent publication (56), we reported that chronic but not acute treatment of differentiated cultured rat aortic smooth muscle cells with insulin induces a suppression of PTP1B function together with decreased binding of PTP1B to PDGFR, enhanced PDGFR tyrosyl phosphorylation, and enhanced cell motility. Taken together, these findings prompted us to test the hypothesis that insulin suppresses PTP1B levels and function in vivo together with increased neointima formation in vascular injury. To this end, we implemented a rat model of hyperinsulinemia involving direct insulin infusion, and we verified that insulin levels were elevated modestly but significantly, without significant hypertension or hypertriglyceridemia, in agreement with another report (6). In addition, we observed the lack of significant hypoglycemia in nonfasted rats together with insignificant change in plasma cortisol and urinary catecholamine levels. Thus, this model allowed us to test the aforementioned hypothesis in relatively long-term experiments in the absence of potentially confounding factors usually associated with hyperinsulinemia. Moreover, the model we implemented involved relatively mild injury to the carotid artery, allowing for intimal expansion to occur for at least 14 days, as we have previously reported (7).

We report the novel finding that the induction of mild hyperinsulinemia elicits lower total PTP1B activity, in parallel with reduced PTP1B protein levels, in injured rat carotid arteries. Thus, reduction of PTP1B activity levels can be explained by reduced levels of PTP1B protein, which, in turn, is attributable to reduced PTP1B mRNA levels. It is interesting to note that we also observed a reduction of PTP1B specific activity in insulin-treated rats at 14 days but not 7 days after vascular injury, indicating the presence of additional mechanisms that target enzyme specific activity and that regulate the overall function of PTP1B. Given the established importance of PDGF signaling in neointimal expansion (14, 17, 22, 54), we also measured the effect of insulin treatment on PDGFR

![Fig. 4. Insulin enhances PDGF receptor (PDGFR) phosphorylation. Lysates obtained 7 days (A and B) or 14 days (C and D) after injury were probed via Western blot analysis. A and C: Western blots; B and D: summary of experiments. IP, immunoprecipitation; Pyr-751, phosphorylated Tyr751. Data are means ± SE of 3 experiments, with each experiment using tissue pooled from 3 rats in the control or insulin treatment category (total number of rats was 18 in each category). *P < 0.05 compared with values of control-injured arteries by ANOVA followed by Fisher’s test.](http://ajpheart.physiology.org/)
phosphorylation and found that insulin induced a marked increase of PDGFR phosphorylation at 14 days but not 7 days after injury. We surmise that the time course for PDGFR dephosphorylation, in which receptor dephosphorylation occurs at the time point of 14 days but not 7 days, can be explained by the finding that both the levels and specific activity of PTP1B are decreased at the time point of 14 days but not 7 days. Moreover, as we have previously reported (7), our model of moderate vascular injury allows neointimal expansion to continue between 7 and 14 days after injury, thus setting the background for the manifestation of the effect of insulin on neointima formation at the 14-day time point. These findings and those of a previous study (7) showing that interference with PTP1B signaling induces major neointimal expansion are consistent with the view that reduced PTP1B function can explain insulin-induced amplification of neointima formation via enhanced PDGFR phosphorylation.

A previous study (41) has reported that FGF is another growth factor that plays a prominent role in vascular injury-induced neointimal expansion, and we (7) have found that PTP1B also targets FGF signaling, at least in cultured cells, and that interference with PTP1B function enhances the motility of cultured cells induced by FGF2. Thus, it is possible that amplification of FGF receptor signaling by insulin-induced suppression of PTP1B function may also play a role in neointimal expansion induced by hyperinsulinemia, although the present study does not address this aspect. It should also be noted that the present model of hyperinsulinemia was not associated with significant nonfasting hypoglycemia, making it unlikely that the results could be attributed to insulin-induced hypoglycemia.

A strength as well as a limitation of the present study is the use of a model of hyperinsulinemia not manifesting altered pathophysiological variables usually associated with more complicated genetic or nongenetic models of hyperinsulinemia, e.g., those involving metabolic syndrome and/or type 2 diabetes. The effect(s) of other factors usually associated with hyperinsulinemia, such as hypertension, hypertriglyceridemia, elevated glucocorticoid and catecholamine levels, etc., on the hyperplastic effect of insulin reported here is unknown, and it will therefore be desirable to extend the present findings to more complex experimental models manifesting metabolic syndrome and type 2 diabetes. Finally, we speculate that PTP1B may also play an important role in human vascular remodeling, but this topic also remains to be investigated.

ACKNOWLEDGMENTS


GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-63886 and HL-72902.

REFERENCES


19. Frangioni JV, Beahm PH, Shifrin V, Jost CA, Neel BG. The nontransmembrane tyrosine phosphate PTP-1B localizes to the endoplasmic
ENHANCED NEOINTIMA FORMATION INDUCED BY INSULIN

H139


