A Central Role of PTP1B in Hyperinsulinemia-Enhanced IL-6 Signaling in Dedifferentiated Vascular Smooth Muscle Cells

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Abstract

Hyperinsulinemia is associated with an increased risk of vascular restenosis after angioplasty. As a major pro-inflammatory cytokine, interleukin-6 (IL-6) induces motogenic effects on vascular smooth muscle cells. Attenuation of vascular injury-induced neointima thickening was observed by blocking STAT3 tyrosine phosphorylation, which is a key component of IL-6 signaling. A non-receptor protein tyrosine phosphatase, PTP1B, plays a counter-regulatory role in injury-induced neointima formation by inhibiting platelet-derived growth factor (PDGF)-induced smooth muscle cell migration and proliferation. However, the role of IL-6, in association with hyperinsulinemia, with an increased risk of vascular restenosis and the involvement of PTP1B in this process has not been studied. Using subcultured (passages 5-9) smooth muscle cells isolated from rat aortae, we found that: 1) chronic insulin treatment potentiated IL-6-induced smooth muscle cell migration and STAT3 tyrosine phosphorylation; 2) chronic insulin dose-dependently suppressed the baseline expression of endogenous PTP1B; 3) overexpressing wild-type PTP1B significantly attenuated whereas C215S-PTP1B enhanced IL-6-induced STAT3 phosphorylation and smooth muscle cell migration. The aforementioned results suggest that inhibition of baseline expression of PTP1B and subsequent potentiation of IL-6-stimulated vascular smooth muscle migration may serve as a potential mechanism for increased risk of vascular restenosis after angioplasty in patients with insulin resistance.

Keywords: Hyperinsulinemia; IL-6; PTP1B; Smooth muscle cells; Migration

Hyperinsulinemia, a major feature of type 2 diabetes and the metabolic syndrome, is believed to be highly associated with the occurrence of atherosclerosis and vascular restenosis [1-3]. In humans with insulin resistance, the frequency of restenosis after coronary angioplasty is significantly higher than those with normal insulinemia [4]. Animals with hyperinsulinemia show increased neointima formation caused by vascular injury via potentiating smooth muscle cell migration and proliferation [5-7]. The application of insulin sensitizers, such as synthetic thiazolinediones (STD), significantly reduces carotid artery intima/media thickness in patients with type 2 diabetes [8-10] and in animals with induced carotid injury [11-14]. However, the underlying mechanisms are not well understood. Recurrent stenosis after angioplasty is the result of increased smooth muscle cell proliferation in the media layer and migration to the intima in the wall of the vasculature. It is well established that vascular inflammation in response to angioplasty-induced injury is the initiator of increased neointima formation. Several experimental and clinical observations indicate that up regulation of pro-inflammatory cytokines in activated SMCs contributes to angioplasty-induced restenosis through promoting vascular smooth muscle cell migration and proliferation, a manifestation of an inflammatory wound healing process occurring in injured vessels [15-17]. Of the many cytokines involved in injury-induced inflammation, IL-6 is the major pro-inflammatory cytokine that contributes to vascular injury-induced neointima thickening [18]. IL-6 is expressed and synthesized by a variety of cell types implicated in intimal hyperplasia. These include endothelial cells, macrophages, and smooth muscle cells. IL-6 induces a motogenic effect on vascular smooth muscle cells [19,20]. A significant correlation between the changes of IL-6 concentrations in the coronary circulation after PTCAs and the degree of restenosis has been observed [21]. Monitoring variations in IL-6 in IL-6 has been proposed as an inflammatory marker to detect the early stage of cardiovascular diseases in order to develop a beneficial strategy to prevent the progression of the diseases. It was also reported that IL-6 induced the expression of acute phase proteins and several other cytokines and growth factors[22], suggesting that IL-6 may serve as a major initiator to trigger inflammation cascade responses that later lead to restenosis. One of the signal transduction pathways that mediates IL-6-mediated cellular responses is gp130/JAK/STAT cascade [19]. The formation of IL-6 and the IL-6 receptor complex promotes the recruitment of gp130, followed by activation of Janus kinase (JAK). Activation of JAKs leads to tyrosine phosphorylation of gp130 and recruitment of STATs. STATs, in turn becomes phosphorylated and dimerized, and translocate into the nucleus, where it binds to the target genes and regulates gene transcription and protein expression [19], leading to cell migration and proliferation. Inhibition of excessive IL-6 signaling by blocking the gp130/JAK/STAT pathway has become a promising intervention to reduce inflammation. A recent study reported that vascular injury increased STAT phosphorylation and that blockade of gp130/STAT, signaling decreased balloon injury-induced STAT phosphorylation, reduced smooth muscle cell migration from media to intima, and attenuated neointima formation [23], suggesting that IL-6 plays a pivotal role in vascular injury-induced neointima formation by activating gp130/STAT, pathway. PTP1B is a non-receptor protein tyrosine phosphatase that serves as a negative regulator in several signal transduction pathways. PTP1B upregulation was observed in a rat carotid artery injury model [24,25]. Transfection of PTP1B in cultured vascular smooth muscle cells revealed inhibition of motogenic...
effect in response to platelet-derived growth factor (PDGF) [26,27], by abrogation of PDGF-induced receptor tyrosine phosphorylation. Over-expression of dominant negative PTP1B significantly potentiated vascular injury-caused neointima formation [27]. These findings indicate that PTP1B plays a counter-regulatory role in injury-induced intimal thickening by attenuating PDGF-induced smooth muscle cell migration and proliferation. Although it is well established that an extensive inflammatory reaction is associated with insulin resistance-related vascular complication [28,29], that hyperinsulinemia increased the risk of vascular restenosis after angioplasty, and that vessel wall inflammation is the initial responder in vascular injury-induced restenosis, the role of PTP1B in hyperinsulinemia-induced high frequency of vascular re- stenosis and the involvement of PTP1B in regulation of pro-inflammatory signaling have never been studied. Our current study was designed to test the hypothesis that chronic hyperinsulinemia enhances vascular injury-induced restenosis by suppressing the expression of endogenous PTP1B and subsequently potentiating the motogenic effect of IL-6.

Materials and Methods

Smooth muscle cell cultures were prepared from adult male Sprague-Dawley rats. Rats were sacrificed by inhalation of CO2. The protocol for animal use was approved by the A.T. Still University Animal Care and Use Committee by complying with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, NIH Publication No. 86-23, Revised 1996).

Materials

Male Sprague-Dawley rats were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA). DMEM and DMEM:Ham’s F-12 (1:1) medium and fetal bovine serum were obtained from Fisher Scientific (Pittsburgh, PA); porcine pancreatic elastase and collagenase were obtained from Worthington Biochemical (Lakewood, NJ); soybean trypsin inhibitor, BSA (fraction V), bovine pancreatic insulin, protease inhibitor cocktail, mouse IgG2a, human recombinant interleukin-6, and all the chemicals were purchased from Sigma (St. Louis, MO); antibodies directed against STAT3 and phospho-STAT3 (Tyr705) were obtained from Cell Signaling (Boston, MA); PTP1B monoclonal antibody was purchased from BD Biosciences (San Jose, CA); protein G-Sepharose beads were from GE (Piscataway, NJ); and adenovirus encoding EGFP (enhanced green fluorescent protein), human sequence wild-type PTP1B, or dominant negative PTP1B (C215S-PTP1B) were kindly donated by Dr. Aviv Hassid (University of Tennessee Health Science Center, Memphis, TN).

Cell culture

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortae of 100-125-g male Sprague-Dawley rats by enzymatic dissociation following the published procedure [30] and grown in DMEM/F12 supplemented with 10% (v/v) heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO₂ atmosphere. The experiments were performed by using subcultured vascular smooth muscle cells between 5 and 9 passages by following a previously published paper [23]. The rationale for using subcultured smooth muscle cells isolated from rat aorta is based on the evidence that there are extensive phenotypic similarities among subcultured aortic smooth muscle cells and the cells in neointima. Several studies revealed that the smooth muscle cells isolated from neointima showed morphologically and functionally similar characteristics to the dedifferentiated smooth muscle cells [31,32]. Phenotypic changes of smooth muscle cells from a differentiated to a more immature (or dedifferentiated) state after vascular injury allow the cells to replicate and expand [33-35]. Furthermore, smooth muscle cells in intima express the genes that represent the characteristics of developmental stages of smooth muscle cells [36,37]. The changes of growth patterns and gene expression, the major events that cause neointima formation after vascular injury, are believed to be associated with the loss of intracellular growth control [38]. These data indicate that subcultured cells are the appropriate model to mimic the cells in both media and neointima in injured vessel wall.

Measurement of cell motility

VSMC motility was measured by cell wounding as described previously [27]. Briefly, confluent cells were subjected to a scratch of about ~20 μm width made with a 10 μl sterile pipette tip. Pictures were taken before and after 24 hour treatment by using a digital camera from Scion Corporation (Frederick, MD). Images were analyzed by using Image J software. Cell migration is expressed as distance covered by cells during 24 hour incubation. 5 mM of hydroxyurea was used to prevent cell proliferation [39].

STAT₃ phosphorylation measurement

At the end of each treatment, cells were lysed on ice in a cold RIPA buffer (PBS, 1% Igepal CA630, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.2) containing a protease inhibitor cocktail (1 mM AEBSF, 0.8 μM aprotonin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, 14 μM E64) and 1 mM of sodium selenite. STAT₃ phosphorylation was checked by either immunoprecipitation with anti-STAT₃, followed by probing with anti-phospho-STAT₃, at Tyr 705, or direct western blot probed with phospho-STAT₃, antibody (as indicated in the legend). Samples used to check tyrosine phosphorylation of STAT₃ were resolved by electrophoresis on 0.1% SDS and 10% polyacrylamide gels. Total STAT₃ was checked by stripping and reprobing the membranes with anti-STAT₃, to serve as loading controls. The antigen-antibody complexes were detected using a Chemiluminescence reagent kit (Perkin Elmer Life Science, Boston, MA).

Adenovirus preparation

Adenoviruses expressing EGFP, wild-type PTP1B, or C215S-PTP1B were generously donated by Dr. Aviv Hassid (University of Tennessee Health Science Center). Adenoviral vectors were constructed by using the technology developed by the University of Iowa. This technique allows the generation of totally homogeneous adenoviral vectors that do not require plaque purification. Briefly, relevant cDNAs were subcloned into pShuttle, which serves as a transfer vector that allows homologous recombination with viral backbone DNA. The Vector pShuttle contains an extensive multiple cloning site, making it possible to subclone most cDNAs into a site 3’ from the CMV immediate/early promoter/enhancer with ease. Following preparation of recombinant pShuttle, the vectors were linearized and co-transfected with viral backbone DNA into HEK293 cells using the lipid transfer agent, Fugene-6. Following several passages in 293 cells, sufficient adenovirus was obtained, allowing purification to a high titer via an affinity purification step using a kit from BD Inc. (Franklin Lakes, NJ).
Statistical analysis

The differences between the treatments were analyzed by one way analysis of variance (ANOVA) followed by post hoc test and student t-test. A p value of less than 0.05 was considered as a significant difference. All the experiments were repeated at least three times.

Results

Chronic insulin treatment potentiates IL-6-induced vascular smooth muscle cell migration

To understand the role of IL-6 in insulin-enhanced vascular injury-induced restenosis, we first tested the effect of chronic insulin treatment on IL-6-induced vascular smooth muscle cell migration. It should also be noted that insulin can, by itself, induce cell motility in dedifferentiated cells if it is present at a sufficiently high level. Therefore, for the present experiments, we titrated insulin concentration down to the level (5nM) at which it produced no significant increase in motility in order to avoid a potential confounding effect of altered baseline motility. As shown in (Figure 1) treatment of cells with IL-6 at the concentration of 20ng/ml significantly stimulated vascular smooth muscle cell migration. Pretreatment of the cells with the low concentration of insulin significantly potentiated the motogenic effect of IL-6.

Chronic insulin treatment potentiates IL-6-induced STAT3 phosphorylation in VSMCs

Our next experiment was designed to study the signal transduction pathway that is involved in the potentiation of the IL-6-stimulated motogenic effect in cells treated with insulin. The experiment was carried out by testing the effect of chronic insulin treatment on IL-6-induced tyrosine phosphorylation of STAT3, a key event in IL-6 signaling. As shown in (Figure 2A and 2B) and B, IL-6 stimulated STAT3 tyrosine phosphorylation in a time- and dose-dependent manner. With the IL-6 concentration of 20ng/ml, STAT3 tyrosine phosphorylation peaked at the 30 min time point. Pretreatment with insulin (100nM) for 24 hours significantly potentiated IL-6-induced STAT3 tyrosine phosphorylation (Figure 2C), suggesting that IL-6 may act as a mediator for insulin-enhanced vascular restenosis following angioplasty. We also tested the effect of the low concentration of insulin (5nM) on IL-6-induced STAT3 tyrosine phosphorylation and found that 5nM of insulin produced similar effects on IL-6-stimulated STAT3 phosphorylation as those shown in (Figure 2A and 2B), IL-6 stimulated STAT3 tyrosine phosphorylation.(Figure 2C and 2D), and lysed with RIPA buffer. p-STAT3 was checked by immunoprecipitation with anti-STAT3 and probed with anti-phospho-STAT3 (Tyr705). Membranes were stripped and reprobed with anti-STAT3 to serve as a loading control. Upper panels show the representative Western blot. Graphs show mean±SE of the ratio of phosphorylated STAT3 to total STAT3 from three independent experiments. Data were analyzed by using One-Way ANOVA followed by post hoc test. *P<0.05, **P<0.01, compared to control and insulin. ¥P<0.05 compared to IL-6 alone.
produced by a higher concentration of insulin (Figure 2D). Therefore, subsequent experiments were all carried out by using 100nM of insulin.

Chronic insulin treatment dose-dependently suppresses the baseline expression of endogenous PTP1B in dedifferentiated smooth muscle cells

Our previous results showed that levels of PTP1B are significantly increased after vascular injury and over expression of dominant negative PTP1B potentiates injury-induced neointima formation, indicating that PTP1B plays a counter-regulatory role in vascular injury-induced neointima formation. A recent study found that chronic insulin treatment attenuates PDGF-induced, but not baseline, expression of PTP1B in differentiated (primary) cultured vascular smooth muscle cells [5]. The current experiment was designed to test if chronic insulin treatment suppresses the baseline expression of endogenous PTP1B in dedifferentiated (passages 5-9) cultured vascular smooth muscle cells (cells in media and intima of injured arteries), thus promoting the mototetic effect of IL-6. As shown in (Figure 3), chronic insulin treatment of vascular smooth muscle cells dose-dependently suppressed the baseline expression of endogenous PTP1B. The inhibition reached plateau at the concentration of 20nM, suggesting that the responses of differentiated and dedifferentiated smooth muscle cells in response to chronic insulin treatment are different. Inhibition of endogenous PTP1B expression in dedifferentiated vascular smooth muscle cells may play a crucial role in insulin-enhanced vascular injury-induced restenosis because of the extensive phenotypic similarities between subcultured aortic smooth muscle cells and the cells in neointima.

Over expressing wild-type PTP1B attenuates whereas dominant negative (C215S-PTP1B) potentiates IL-6-stimulated smooth muscle cells migration

Our next experiments were designed to test if attenuation of endogenous PTP1B expression is necessary and/or sufficient to explain the augmentation of IL-6-stimulated smooth muscle cell migration caused by chronic insulin treatment. The experiments were performed by examining the effect of wild-type PTP1B and C215S-PTP1B on IL-6-stimulated cell migration by transfecting cells with wild-type and C215S-PTP1B (catalytically essential cystine at 215 is mutated to serine) followed by stimulation with IL-6. As shown in (Figure 4A and B), overexpressing wild-type PTP1B (WT-1B) significantly attenuated the motogenic effect of IL-6 whereas expressing C215S-PTP1B (CS-1B) potentiated IL-6-induced cell migration, indicating that downregulation of endogenous PTP1B may play an important role in insulin-enhanced motogenic effect of IL-6.

Over expressing wild-type PTP1B suppresses whereas C215S-PTP1B potentiates IL-6-induced STAT3 phosphorylation

The aforementioned results suggest that inhibition of PTP1B expression might contribute to hyperinsulinemia-caused potentiation of IL-6-induced cell migration. Our next experiments were designed to test if these effects were mediated through affecting the key element of IL-6 signaling, STAT, tyrosine phosphorylation. Cells were transfected with adenovirus expressing EGFP (control) or wild-type PTP1B or C215S-PTP1B, followed by the stimulation with IL-6. As shown in (Figure 5A and B), wild-type PTP1B significantly attenuated whereas C215S-PTP1B enhanced IL-6-induced STAT3 phosphorylation at the tyrosine 705, suggesting that inhibition of PTP1B expression is the key in augmentation of IL-6-mediated signaling caused by hyperinsulinemia.

Figure 3: Chronic insulin treatment suppresses baseline expression of PTP1B in dedifferentiated vascular smooth muscle cells. Cells were incubated with serum-free medium for 24 hours followed by stimulation with different concentrations of insulin (5, 10, 20, 50, 100nM) for 24 hours. PTP1B protein levels were checked by Western blot directed against PTP1B. Membranes were reprobed with anti-α-actin to serve as a loading control. Upper panel shows the representative Western blot. Graph shows the mean±SE of the ratio of PTP1B to α-actin from three independent experiments. Data were analyzed by using One-Way ANOVA followed by post hoc test. *P<0.05, compared to control.

Figure 4: Wild-type PTP1B attenuates whereas dominant negative (C215S-PTP1B) potentiates IL-6-stimulated smooth muscle cells migration. Cells were transfected with control virus expressing enhanced green fluorescent protein (EGFP) or with virus expressing wild-type (WT-1B, Figure 4A) or dominant negative PTP1B (CS-1B, Figure 4B) at multiplicity of infection values of 10—15 for 24 hours. After viruses were removed, cells were further incubated for 24 hours to allow time for PTP1B expression. Cells were then treated with 20ng/ml of interleukin-6 for 24 hours after wounding. Upper panels show the representative Western blot indicating the expression levels of PTP1B. Graphs show the migration distance (mean±SE) from three independent experiments before and after IL-6 treatment. Data are analyzed by using one-way ANOVA followed by post hoc test. **P<0.01, compared to control, ##P<0.01 compared to IL-6 alone.
Figure 5: Wild-type PTP1B suppresses whereas C215S-PTP1B potentiates IL-6-induced STAT3 phosphorylation. Cells were transfected with adenovirus expressing enhanced green fluorescent protein (EGFP) or with adenovirus expressing wild-type (Figure 5A) or C215S PTP1B (Figure 5B) at multiplicity of infection values of 10—15 for 24 hours. After viruses were removed, cells were further incubated for 24 hours to allow time for PTP1B expression. Cells were then stimulated with 20 ng/ml of IL-6 for 30 min. p-STAT3 was checked with western blot by probing with anti-phospho-STAT3 (Y705). The membrane was stripped and reprobed with anti-STAT3 to serve as a loading control and incubated with anti-STAT3 to check the overexpression levels. Upper panels show the representative Western blot indicating the expression levels of PTP1B. Graphs show mean±SE of the ratio of phosphorylated STAT3 to total STAT3 from four independent experiments. Data were analyzed by using One-Way ANOVA followed by post hoc test. **P<0.01, compared to control, ##P<0.01, compared to IL-6 alone.

Discussion

The novel findings of this report are: 1) chronic insulin treatment potentiated IL-6-induced smooth muscle cell migration and STAT3 tyrosine phosphorylation; 2) chronic insulin dose-dependently suppressed the baseline expression of endogenous PTP1B in dedifferentiated vascular smooth muscle cells; 3) over expressing wild-type PTP1B significantly attenuated, whereas C215S-PTP1B enhanced, IL-6-induced STAT3 phosphorylation; 4) expressing wild-type PTP1B drastically attenuated, whereas C215S-PTP1B augmented, IL-6-stimulated smooth muscle cell migration, suggesting that enhanced IL-11 signaling, mediated by down regulation of PTP1B expression, may contribute to increased risk of vascular restenosis after angioplasty in patients with hyperinsulinemia.

It is well established that interactions between inflammatory cells, endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and extracellular matrix (ECM), and subsequent increased release of cytokines play pivotal roles in vascular injury-induced restenosis by promoting smooth muscle cell growth and migration leading to intima thickening. As a major inflammatory cytokine, IL-6 is one of the early responders after vascular injury. The involvement of IL-6 in vascular injury-induced neointima thickening is well established. Elevated plasma concentration of IL-6 was observed in patients immediately after angioplasty as well as in injured arteries of animal models [40-42]. Blockade of IL-6-stimulated signaling has produced significant reduction of vascular injury-induced neointima formation [23]. However, little is known about the role of IL-6 in insulin-enhanced neointima formation after vascular injury. Our current data show that chronic insulin treatment significantly potentiates IL-6-induced smooth muscle cell migration and STAT3 tyrosine phosphorylation, suggesting that increased IL-6-induced signaling may play an important role in augmentation of vascular injury-induced neointima formation caused by hyperinsulinemia.

The most recent studies found that chronic insulin treatment of differentiated vascular smooth muscle cells significantly suppressed PDGF-stimulated but not the baseline expression of endogenous PTP1B. Rats with hyperinsulinemia showed attenuation of injury-induced PTP1B expression compared to those with normal insulinemia [5,7], suggesting that inhibition of stimulated expression of PTP1B may play an important role in chronic hyperinsulinemia-enhanced neointima formation after vascular injury. Our current results show, for the first time, that insulin dose-dependently suppresses the baseline expression of endogenous PTP1B in dedifferentiated vascular smooth muscle cells. We also found that wild-type PTP1B attenuates, whereas C215S-PTP1B potentiates, IL-6-induced cell migration and STAT3 phosphorylation, suggesting that attenuation of PTP1B expression in dedifferentiated vascular smooth muscle cells (cells in media and intima of injured arteries) may serve as a key factor in increased risk of vascular restenosis in patients with hyperinsulinemia by potentiating the mitogenic effect of IL-6 as well as other growth factors.

In conclusion, the present findings indicate that inhibition of baseline expression of PTP1B in dedifferentiated vascular smooth muscle cells and subsequent potentiation of IL-6-stimulated smooth muscle migration together may serve as a potential mechanism for increased risk of vascular restenosis after angioplasty in patients with insulin resistance.

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