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PROTEIN PHOSPHATASE 1B IN THE SOLITARY TRACT NUCLEUS IS NECESSARY FOR NORMAL BAROREFLEX FUNCTION

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Abstract

Despite positive metabolic effects, genetic deletion of protein phosphatase 1b (PTP1b) results in sympathetically-mediated elevations in arterial pressure (AP) in mice. Since several PTP1b-regulated peptides also impair the baroreflex sensitivity (BRS) for control of heart rate (HR), we hypothesized that PTP1b actions in the solitary tract nucleus (NTS) participate in the maintenance of resting baroreflex function. To test this hypothesis, we performed acute bilateral microinjection of an allosteric PTP1b inhibitor (100 nM/120 nL) in the NTS of urethane/chloralose anesthetized Sprague-Dawley rats and assessed the BRS, responses to cardiac vagal chemosensitive fiber activation and resting AP and HR before and after the injection. PTP1b inhibition impaired the BRS for bradycardia ($n=6$; 0.93 ± 0.14 baseline versus 0.48 ± 0.04 at 10 min versus 0.49 ± 0.04 msec/mmHg at 60 min; $p < 0.01$), with no significant effect on the BRS for tachycardia (0.30 ± 0.16 baseline versus 0.24 ± 0.08 10 min versus 0.24 ± 0.12 msec/mmHg at 60 min). The reduced BRS for bradycardia was associated with a significant decrease in alpha-adrenergic responsiveness to phenylephrine at 60 min after PTP1b inhibition. Injection of the PTP1b inhibitor in the NTS elicited transient decreases in AP and HR in these animals. However, there was no effect of the inhibitor on depressor or bradycardic responses elicited by activation of cardiac vagal chemosensitive fibers, which converge with baroreceptor afferents in the NTS. These results suggest that PTP1b actions within the NTS may be a novel molecular mechanism for preservation of resting baroreflex function and provides further evidence for deleterious cardiovascular effects associated with PTP1b inhibition.

Keywords

phosphatase; transgenic rats; solitary tract nucleus; baroreflex sensitivity

Introduction

Reversible protein phosphorylation by tyrosine kinases or dephosphorylation by tyrosine phosphatases is a major regulatory mechanism underlying numerous intracellular signaling

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events. Protein phosphatase 1b (PTP1b), a member of the protein tyrosine phosphatase family, limits the activation of various intracellular kinase signaling pathways through direct dephosphorylation of tyrosine residues on receptors and/or signaling components.(1) Recent studies provide evidence that PTP1b may play a role in energy metabolism as genetic deletion of PTP1b in mice is associated with lower fat mass, protection against diet-induced obesity and increased insulin and leptin sensitivity.(2;3) This phenotype is recapitulated by neuron-specific deletion of PTP1b providing evidence for primary metabolic effects of the phosphatase in the central nervous system. Indeed, PTP1b is widely expressed in the brain with increased levels in hypothalamic regions critical to regulation of energy metabolism in animal models of obesity, aging and acute inflammation.(3–6) Furthermore, hypothalamic reduction of PTP1b improves insulin and leptin resistance in obese animals (5). The beneficial effects of PTP1b deletion on energy balance implicate inhibitors of this phosphatase as attractive targets in conditions associated with insulin and leptin resistance including obesity and type II diabetes.

Despite positive metabolic actions, genetic deletion of PTP1b in mice results in elevations in arterial pressure (AP) and enhanced whole body sympathetic tone (7), consistent with the sympathoexcitatory and systemic pressor actions of peptides known to be dampened by PTP1b expression including angiotensin II and leptin (8). These peptides also impair baroreflex sensitivity (BRS) for control of heart rate (HR) (9;10), an important marker of parasympathetic function mediated at the level of the solitary tract nucleus (NTS) in the dorsomedial medulla (11). Impairments in the BRS are observed in numerous cardiovascular diseases, resulting in an imbalance in autonomic outflow and providing a permissive role for chronic adrenergic activation.(11;12) Whether PTP1b is expressed in the NTS and contributes to modulation of BRS for control of HR in this brain region has not been investigated. Although increased PTP1b levels are associated with reduced metabolic sensitivity, endogenous levels of the phosphatase may be necessary for normal BRS. Conversely, inhibition of PTP1b may allow for unrestrained tyrosine kinase signaling to occur, resulting in baroreflex dysfunction.

Thus, in the present study we tested the hypothesis that PTP1b actions within the NTS are necessary for the maintenance of normal baroreflex function. We performed NTS microinjection of an inhibitor of PTP1b in Sprague-Dawley rats and assessed for changes in BRS, resting AP and HR and responses to activation of cardiac vagal chemosensitive fibers. Since genetic deletion may result in unknown compensatory mechanisms or programmed events during development, we used a cell-permeable inhibitor of PTP1b allowing acute interruption of the pathway. This small molecule inhibitor reversibly binds PTP1b with high affinity at an allosteric site that is not conserved among phosphatases and stabilizes the inactive conformation.(13) This compound has been extensively characterized *in vitro* with high selectivity for PTP1b over closely related proteins and phosphatases.(13;14) In addition, a recent study provides evidence for functional efficacy and specificity of this inhibitor in the central nervous system of rats.(6) Using this inhibitor, the present study provides evidence that PTP1b actions in the NTS may serve as a protective molecular mechanism to preserve resting baroreflex function.

Methods

All procedures were approved by the Institutional Animal Care and Use Committee and have been published in detail in recent publications. (9;15;16)

Animals

Experiments were performed in 3 to 5 month-old male Hannover Sprague-Dawley rats obtained from the Hypertension and Vascular Research Center Transgenic Animal Facility,

Wake Forest University School of Medicine, Winston-Salem, NC. Animals were housed in humidity- and temperature-controlled rooms in group cages (12-hour light/dark cycle) with free access to standard rat chow and water.

Surgical Procedures

Rats were anesthetized by intraperitoneal administration of combination urethane-chloralose (750 mg and 35 mg per kg, respectively) with supplemental intravenous doses given as needed. Polyethylene-50 catheters were inserted into the femoral artery and vein for cardiovascular parameters measurements and delivery of drugs, respectively. Rats were placed in a stereotaxic frame for surgical exposure of the dorsal medulla oblongata via incision of the atlanto-occipital membrane and subsequent insertion of the microinjection pipette into the NTS. A period of 30 min was allowed after surgical procedures before baseline measurements.

Hemodynamic Measures

Pulsatile and mean AP was monitored, recorded and digitized using a Data Acquisition System (BIOPAC System Inc.; Acknowledge software Version 3.8.1) with HR derived from the AP waveform.

Reflex Testing

An established tool to assess autonomic control of the heart is the use of pharmacologic agents to evoke the BRS, before and after blockade of receptors or signaling pathways within the NTS.(12;17) Thus, the BRS for bradycardia or tachycardia was established by bolus intravenous injection of randomized doses (2, 5 and 10 $\mu\text{g/kg}$ in 0.9% NaCl) of phenylephrine or sodium nitroprusside, respectively. Since several peptides selectively alter the BRS for bradycardia, (9;10) we performed bolus administration of vasoactive drugs, a method more sensitive to parasympathetic alterations in the BRS relative to ramp infusions. (18) Maximum mean AP responses (Δ mean AP, mmHg) and associated reflex changes in HR (ΔHR , bpm) were recorded at each dose of phenylephrine or nitroprusside and ΔHR was converted to changes in pulse interval (ΔPI , msec) by the formula: $60,000/\text{HR}$. The BRS for each animal was determined as the slope of the relationship between changes in mean AP and the corresponding PI generated from the three doses of phenylephrine or nitroprusside, independently. Depressor and bradycardic responses to cardiac vagal chemosensitive fiber activation (CVA) were established by bolus intravenous administration of the serotonin 5-HT₃ receptor agonist phenylbiguanide (10 $\mu\text{g/kg}$ in 0.9% NaCl). All reflex testing was completed within 30 min.

NTS Microinjections

At least 30 min was allowed after baseline measurements before commencing microinjections. Multi-barreled glass micropipettes connected to a syringe were used to perform bilateral microinjection of the PTP1b inhibitor into the NTS [0.4 mm rostral, 0.4 mm lateral to the calamus scriptorius (caudal tip of the area postrema) and 0.4 mm below the dorsal surface] as previously described.(9;16) Following injections, we measured for immediate peak changes in AP and HR. We also retested the BRS at 10 and 60 min and CVA responses at 10 min after injection of the PTP1b inhibitor, with each animal used as its own control.

PTP1b Inhibitor

An allosteric inhibitor of PTP1b 3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide [Calbiochem, Compound 2; Gibbstown, NJ] was dissolved in artificial cerebrospinal fluid (aCSF) and injected in the

NTS at a dose of 100 nM in 120 nL. This dose was derived from a recent study in which the inhibitor was administered centrally in rats and found to be functionally effective.(6) The volume of injectate is consistent with previous studies and is mostly confined to the NTS and without functional effects on surrounding nuclei.(9;10;19;20) We have consistently shown that microinjection of aCSF vehicle has no effect on the BRS, resting AP and HR or responses to CVA in Sprague-Dawley rats.(9;15) Thus, vehicle injections were not repeated in the present study. The pharmacologic properties of this PTP1b inhibitor have been described in detail in previous reports.(13) This inhibitor reduces PTP1b activity *in vitro* and has been widely used to examine the role of PTP1b to cellular processes and insulin signaling.(13;14;21–23) Acute intracerebroventricular administration of this inhibitor restores anorectic responses to leptin administration, without altering food intake, in leptin-resistant aged Sprague-Dawley rats (6) providing functional evidence for *in vivo* efficacy and specificity.

Histology

Following microinjection experiments, brains were removed, frozen on dry ice and sectioned (30 μ M) for localization of microinjection sites. Only injections within the medial NTS at rostro-caudal level –13.3 to –14.0 mm caudal to bregma were included in this study, as recently illustrated.(9) The overall success rate for this study was > 85% with 7 animals receiving microinjections and 1 animal excluded due to a missed injection site.

Localization of PTP1b in the Dorsal Medulla

To establish localization of PTP1b within the dorsal medulla, we excised 1 mm³ sections of tissue from frozen brains obtained from a separate group of conscious Sprague-Dawley rats (n = 6) as previously described.(9;15) These sections contain mostly NTS, but also small portions of area postrema and dorsal motor nucleus of the vagus, corresponding to the expected spread of the PTP1b inhibitor injection. Gene expression of protein tyrosine phosphatase non-receptor 1 (PTPN1), the gene encoding PTP1b,(24) was measured by reverse transcriptase real-time PCR using a probe available from Applied Biosystems (Carlsbad, CA). As previously reported,(25;26) data were quantified as C_t values, where C_t is the threshold cycle at which amplified product is first detected and is defined as relative gene expression for the ratio of PTPN1 to 18S ribosomal RNA control. Protein expression of PTP1b was also measured in dorsal medullary tissue. The tissue was homogenized in PBS [50 mmol/L NaPO₄ (pH 7.2), 100 mmol/L NaCl] and solubilized by adding PBS to the fraction with boiling 3% SDS-10% β -mercaptoethanol. Protein concentration was measured by modification of the Lowry method.(25) Solubilized protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham Hybond-P, GE Healthcare, Piscataway, NJ). Non-specific binding was blocked with blotto and membranes were incubated with a PTP1b primary antibody (1:1,000; Calbiochem, Gibbstown, NJ) followed by an anti-mouse Ig-HRP secondary antibody (1:5000; GE Healthcare, Piscataway, NJ). Chemiluminescence reagents (SuperSignal Femto West, Pierce Biotechnology, Rockford, IL) were added to visualize the immunoreactive bands, which were quantified by densitometry. The gels were stripped and reprobed with an antibody to β -actin (Sigma, St. Louis, MO) as a loading control. Data were expressed as the ratio of PTP1b to β -actin protein levels.(25;26)

Analysis of Data

Values are presented as mean $\pm$ SEM. Comparisons of changes in baroreflex function and resting hemodynamics over time in response to the PTP1b inhibitor were analyzed by repeated-measures ANOVA with post-hoc Student-Newman-Keuls multiple comparisons. Differences in CVA responses at baseline versus 10 min were assessed by paired Student's

t-test. The criterion for statistical significance was $p < 0.05$. Tests were performed using Prism 4.0 and InStat 3 (GraphPad Software).

Results

Resting Mean AP and HR in Response to NTS Injection of the PTP1b Inhibitor

Microinjection of the PTP1b inhibitor within the NTS resulted in a transient decrease in mean AP which peaked at 2 ± 1 min and recovered by 10 min after administration (Figure 1A and B). The PTP1b inhibitor also produced a significant decrease in HR, which was sustained at 10 min, but recovered to baseline levels by 60 min after the injection (Figure 1A and C).

Effect of PTP1b Inhibition on Reflex Function

In pooled data, there was a 52% reduction in the slope of the relationship between the increases in mean AP produced by phenylephrine and the corresponding reflex bradycardia at 10 min after injection of the PTP1b inhibitor (Figure 2A). The BRS was also examined at 60 min after the injection, with a 48% reduction in the slope of the regression observed at this time point (Figure 2A). The mean values from individual Sprague-Dawley rats showed a significant 48% reduction in the BRS after PTP1b inhibition (Figure 2B; $p < 0.01$). The BRS remained impaired by 47% when retested at 60 min after the injection (Figure 2B, $p < 0.01$). There were no changes in α -adrenergic responsiveness at 10 min after PTP1b inhibition relative to baseline (Figure 2C). In contrast, pressor responses to phenylephrine were significantly reduced at 60 min after injection of the PTP1b inhibitor (Figure 2C, $p < 0.05$). There was no significant effect of PTP1b inhibition on the BRS for tachycardia to decreases in AP evoked by nitroprusside in these animals (Figure 2D). PTP1b inhibition also did not alter depressor or bradycardic responses to activation of cardiac vagal chemosensitive afferent fibers in these animals (Figure 3).

Localization of PTP1b in the Dorsal Medulla

Since localization of PTP1b within the NTS has not been reported, we measured for mRNA and protein expression in dorsal medulla tissue sections obtained from a separate group of Sprague-Dawley rats (Figure 4A and B). Gene expression for PTPN1, the gene encoding PTP1b, and PTP1b protein expression were observed in the dorsal medulla of these animals.

Discussion

The goal of this study was to determine whether PTP1b within the NTS contributes to the maintenance of resting baroreflex function. The key findings are that inhibition of PTP1b in the NTS results in: 1) transient decreases in resting AP and HR; 2) significant impairment of the BRS for bradycardia, independent of sustained changes in AP or HR; 3) no significant changes in the tachycardic BRS or responses to activation of cardiac vagal chemosensitive fibers. Furthermore, we provide evidence for PTP1b gene and protein expression within the dorsal medulla, an area integral for central cardiovascular and baroreflex regulation. Collectively, these results suggest a novel role for PTP1b in the modulation of the BRS at the level of the NTS and provide insight into molecular mechanisms participating in baroreflex regulation.

The most prominent finding of our study is the dramatic reduction in the BRS for bradycardia, to levels observed in hypertensive animals, (27;28) in response to PTP1b inhibition. This supports a functional role for PTP1b in brain centers participating in activation of parasympathetic outflow pathways in response to baroreceptor activation. The BRS is typically assessed within 15 min after microinjections and was altered at this time

point in the presence of the PTP1b inhibition. In the present study, changes in resting HR were still evident at 10 min after the injection, which could directly influence baroreflex function. Thus, we retested the BRS at 60 min when hemodynamic measures had returned to baseline levels and observed continued suppression of the baroreflex. This finding confirms that different neural mechanisms are involved in regulation of BRS versus resting hemodynamics.(15;20) Although this PTP1b inhibitor is reported to be reversible, we did not examine the time course for recovery of the BRS. At the present time, we do not know the mechanisms underlying PTP1b modulation of baroreflex function. Potential mechanisms include alterations in the activity of kinases, neurotransmitter receptors (glutamate and GABA), voltage-gated channels or intracellular signaling pathways within the NTS.

The selective impairment of the BRS to increases but not decreases in pressure is consistent with the actions of other peptides in the NTS (9;10;16) and mimics findings in human hypertension (29). Although lower than the bradycardic BRS, the resting level of the tachycardic BRS is within the range of reported values for anesthetized rats.(9;10;16;30) An inherent asymmetry in the HR responses to changes in pressure elicited by vasoactive drugs, with depressed responses to baroreflex deactivation produced by nitroprusside, has been previously observed.(31) Providing evidence for modality-specific actions, the PTP1b inhibitor did not alter responses to activation of cardiac vagal chemosensitive afferent fibers which converge with baroreceptor inputs in the NTS (32). We did not assess responses to different phenylbiguanide doses; however, the dose used is submaximal and has been previously used to show higher and lower responses in transgenic relative to Sprague-Dawley rats.(33) Moreover, the attenuation of the BRS was seen across the dose range of responses for phenylephrine, suggesting the level of vagal activation is not a limiting factor in the effects of the phosphatase inhibition.

While there was no short-term effect, the PTP1b inhibitor reduced pressor responses to phenylephrine at 60 min after the injection. This is consistent with blunted vascular adrenergic responses to phenylephrine and downregulation of vascular α 1-adrenergic receptor expression in PTP1b knockout mice.(7) However, this is opposite to what is expected if the BRS is attenuated, where enhanced pressor effects in response to phenylephrine might occur. Long-term attenuation of the vagal component of BRS associated with the loss of the phosphatase would be expected to play a permissive role in the development of hypertension and favor sympathetic activation. In fact, such a pattern is reported in PTP1b knockout mice, in spite of the reduced responsiveness to phenylephrine observed in these animals. Consistent with the present study, peptides that produce systemic pressor effects and overall attenuation of BRS often elicit transient depressor and bradycardic responses in the NTS.(9;10;34) While the mechanism of the depressor response to PTP1b inhibition is unknown, the acute decreases in pressure caused by angiotensin peptides in the NTS involve glutamate and substance P release.(34)

There are potential limitations in the present study. First, it remains possible that the BRS suppression is mediated by non-specific actions of the PTP1b inhibitor. This seems unlikely given the inhibitor specificity for modification of baroreceptor afferent inputs and the bradycardic BRS, the consistency with long-term genetic deletion of the phosphatase (7), and that the effect is opposite to that of inhibition of the PI3K pathway.(35) Second, these studies were conducted in anesthetized animals; however, we employed an agent widely known for its preservation of autonomic function relative to other anesthetics.(36) Under this anesthesia, we have shown that the level of resting BRS is similar between conscious and anesthetized animals and responses to other pharmacologic inhibitors are not confounded.(37;38) Third, we examined acute, site-specific inhibition of PTP1b within the NTS. Further studies are needed to address whether chronic administration, either peripheral or central, results in baroreflex dysfunction and subsequent elevations in AP.

Conclusions

The present results provide evidence that inhibition of PTP1b, at least at the level of the NTS, is accompanied by baroreflex dysfunction. Impairments in the BRS are independently associated with increased cardiovascular risk and may provide a permissive role for increases in AP.(11;12) Thus, while PTP1b inhibition may be effective to improve metabolic function in insulin and leptin-resistant populations especially at hypothalamic sites of action, reduced PTP1b actions may also lead to deleterious cardiovascular outcomes including baroreflex dysfunction. These findings may provide insight into molecular mechanisms underlying baroreflex dysfunction in conditions associated with phosphatase dysregulation including obesity, type II diabetes and aging and provide further caution for the systemic use of PTP1b inhibitors in the treatment of these conditions. Since there is limited knowledge regarding signaling mechanisms involved in baroreflex modulation, the present findings also provide important insight into unraveling pathways by which the BRS is mediated.

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Figure 1. Hemodynamic responses to NTS inhibition of PTP1b

A, Representative arterial pressure (AP) and heart rate tracings in response to PTP1b inhibition within the NTS. B, Microinjection of the PTP1b inhibitor resulted in a transient decrease in resting mean AP which peaked at 2 ± 1 min and recovered to baseline levels at 10 min after the injection. C, Inhibition of PTP1b within the NTS produced a significant decrease in resting heart rate, an effect that was sustained at 10 min, but recovered to baseline by 60 min after the injection. $n = 6$, * $p < 0.05$ versus baseline and 60 min

Figure 2. Effects of PTP1b inhibition on baroreflex sensitivity and α -adrenergic responsiveness

A, The slope of the relationship between increases in mean arterial pressure (AP) produced by phenylephrine and corresponding reflex bradycardia [expressed as pulse interval (PI)] shows a reduction in the linear regression slope after NTS injection of the PTP1b inhibitor [0.88 ± 0.28 baseline versus 0.42 ± 0.13 at 10 min versus 0.46 ± 0.09 msec/mmHg at 60 min ($r^2 = 0.40 - 0.67$)] in pooled data from Sprague-Dawley rats. B, The mean baroreflex sensitivity (BRS) values shows that PTP1b inhibition significantly impaired the BRS for bradycardia at 10 and 60 min after the injection. C, Changes in mean AP in response to intravenous phenylephrine were not altered at 10 min after injection of the PTP1b inhibitor. In contrast, phenylephrine produced significantly lower increases in mean AP at 60 min after the injection suggesting reduced α -adrenergic responsiveness at this time point. D, There was no significant effect of PTP1b inhibition on the BRS for tachycardia evoked by nitroprusside in these animals. $n = 6$, * $p < 0.05$ versus baseline, # $p < 0.05$ versus baseline and 10 min

Figure 3. Responses to cardiac vagal chemosensitive fiber activation

Microinjection of the PTP1b inhibitor within the NTS did not significantly alter depressor (A) or bradycardic (B) responses to activation of cardiac vagal afferent chemosensitive fibers evoked by intravenous phenylbiguanide in anesthetized Sprague-Dawley rats. n = 6

Figure 4. Localization of PTP1b in the dorsal medulla of Sprague-Dawley rats

A, Reverse transcription real-time PCR revealed mRNA expression for PTPN1, the gene encoding PTP1b, in brain dorsal medulla of Sprague-Dawley rats (n = 6). Results are expressed as relative gene expression of PTPN1 to the 18S ribosomal RNA control. B, PTP1b protein expression in dorsal medulla was assessed by western blot analysis as shown in the representative gel. Protein expression was quantified by densitometry as the ratio of PTP1b to the β -actin loading control (n = 6).