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Disrupting protein tyrosine phosphatase σ does not prevent sympathetic axonal dieback following myocardial infarction

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Abstract

The neuronal receptor protein tyrosine phosphatase receptor σ (PTP σ) inhibits axonal extension upon binding to chondroitin sulfate proteoglycans (CSPGs) in scar tissue. We recently demonstrated that modulating or deleting PTP σ promoted re-innervation of the CSPG-containing cardiac scar after ischemia-reperfusion (I-R). However, it remains unknown if the lack of PTP σ or early treatment with the PTP σ modulator, intracellular sigma peptide (ISP), prevents the initial injury-induced axonal dieback. To address this, we carried out I-R in PTP σ $-/-$ mice or control littermates treated with ISP or vehicle immediately at the time of I-R, and then assessed sympathetic innervation of the scar and surrounding myocardium 3 days later. Vehicle-treated WT controls displayed sympathetic denervation within the scar and viable tissue adjacent to the scar, as well as distal myocardium farther from the scar. PTP σ $-/-$ and ISP-treated animals also displayed denervation of the scar and adjacent tissue, but regions distal to the scar were innervated normally. This suggests that PTP σ does not mediate axonal dieback but its disruption enhances axonal regrowth in the heart. CSPG digestion alters the macrophage response to prevent axonal dieback in spinal neurons, so we investigated whether targeting PTP σ might alter the macrophage response in the heart. The macrophage response after I-R was similar in vehicle and ISP-treated groups. Mice lacking PTP σ trended toward an increased M2 response, but were not significantly different than the other groups. These data suggest that PTP σ is not involved in axonal dieback or the early macrophage response following cardiac I-R.

Keywords

protein tyrosine phosphatase sigma; intracellular sigma peptide; chondroitin sulfate proteoglycans; sympathetic nervous system; macrophage; myocardial infarction; ischemia-reperfusion; peripheral nerve regeneration

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Introduction

The neuronal receptor protein tyrosine phosphatase sigma (PTP σ) restricts axonal extension upon binding to chondroitin sulfate proteoglycans (CSPGs) in the extracellular matrix (Dyck and Karimi-Abdolrezaee, 2015). A diverse family of CSPGs differing in core proteins and attached chondroitin sulfate side chains are produced in scar tissue following injuries such as spinal cord trauma (Dyck and Karimi-Abdolrezaee, 2015) and cardiac ischemia-reperfusion (I-R) (Gardner and Habecker, 2013). Axons expressing PTP σ bind to these CSPGs and “stall,” thereby failing to re-innervate the scar (Dyck and Karimi-Abdolrezaee, 2015; Filous et al., 2014). In the heart, this axonal stall results in sustained denervation of the scar and border zone (Gardner and Habecker, 2013), and increases the propensity for potentially fatal ventricular arrhythmias (Gardner et al., 2015).

Employing the bacterial enzyme, chondroitinase ABC (ChABC), to digest inhibitory CSPG side chains attenuates the immediate progressive degeneration known as axonal dieback (Busch et al., 2009) and enhances sympathetic axonal extension through CSPG-enriched substrates (Gardner and Habecker, 2013). The mechanisms by which CSPGs regulate axonal dieback remain incompletely understood but “M1” and “M2” macrophages may be involved. M1 macrophages are inflammatory, deleterious, and dominate during early injury phases, while M2 macrophages dominate at later intervals and play a more beneficial, reparative role (Gensel and Zhang, 2015). ChABC has been shown to promote M2 macrophage polarization, which contributes to enhanced axonal extension and reduced axonal dieback in spinal injury models (Didangelos et al., 2014; Gensel and Zhang, 2015).

Conversely, manipulating PTP σ directly as opposed to digesting CSPGs has also proven effective at restoring nerve regeneration through scar tissue. Recently we demonstrated that lack of PTP σ led to re-innervation of the cardiac scar and border zone after I-R (Gardner and Habecker, 2013; Gardner et al., 2015). However, whether this normal innervation is achieved by increased axonal extension or from attenuated axonal dieback remains unknown. Indeed, while numerous reports have shown efficacy with ChABC restoring axonal extension *in vivo* from acute or delayed treatment following spinal injury, axonal innervation assessments were made many days (or weeks) following initial treatments (Dyck and Karimi-Abdolrezaee, 2015); hence, the distinctions between axonal extension versus attenuated axonal dieback following injury are not clear. Importantly, the direct role of PTP σ in axonal dieback has not been tested. Here we provide evidence that modulation or deletion of PTP σ does not prevent axon dieback after cardiac ischemia-reperfusion, but does enhance axon innervation in regions distal to the scar.

Material and methods

Animals

BALB/c mice heterozygous for PTP σ (*ptprs* $+/-$) were provided by Dr. Michel Tremblay at McGill University (Elchebly et al., 1999). PTP σ $-/-$ animals were created by breeding a maintained colony of heterozygotes; $+/+$ BALB/c mice served as WT controls. All mice were kept on a 12 hour light-dark cycle with access to food and water *ad libitum*. Mixed-sex and age-matched littermates between 12 and 18 weeks of age were used for surgeries. All

procedures were approved by the Oregon Health and Science University (OHSU) Institutional Animal Care and Use Committee and comply with the Guide for the Care and use of Laboratory Animals published by the National Academies (8th edition).

Cardiac ischemia-reperfusion (myocardial infarction)

Myocardial infarction (MI) was induced by ischemia-reperfusion (I-R) as previously reported (Gardner and Habecker, 2013). Briefly, the left anterior descending coronary artery was reversibly ligated for 45 minutes and then reperused by releasing the ligature. Occlusion was confirmed by sustained S-T wave elevation and regional cyanosis. Reperfusion was confirmed by the return of color to the ventricle distal to the ligation and reperfusion arrhythmia. Core body temperature was monitored by a rectal probe and maintained at 37°C, and a two-lead electrocardiogram was monitored. Sham controls underwent an identical procedure except for the left anterior descending coronary artery ligation.

Treatment groups

Immediately following MI, animals were injected intraperitoneally with 20 µg of the PTP α modulator, Intracellular Sigma Peptide (ISP) (Lang et al., 2015) (4% DMSO, 96% saline: 100 µl volume). Additional injections were given 24 and 48 hours later, and hearts were removed 3 days after surgery. This dose of ISP is sufficient to promote axon regeneration after spinal cord injury or myocardial infarction (Gardner et al., 2015; Lang et al., 2015).

Tissue preparation and immunohistochemistry

On day 3 post-MI, mice were anesthetized with 4% isoflurane and the hearts were removed. The hearts were rinsed in saline, trimmed of the atria and extraneous tissue, and immediately fixed in 4% paraformaldehyde at room temperature for 1 hour. Hearts were rinsed 3 times in PBS and then cryo-protected in 30% sucrose overnight at 4°C. The hearts were mounted in OCT and sectioned at 10 µm thickness for immunohistochemistry as previously described (Gardner and Habecker, 2013). Sections were collected near the beginning, middle, and end of the infarct zones along the transverse z-axis from base towards apex.

Tyrosine hydroxylase (TH, rabbit IgG, 1:1000, Invitrogen) was used as a marker for sympathetic nerves in the heart, and fibrinogen (sheep IgG, 1:500; Serotec) was used to visualize the infarcted tissue; images using the DAPI filter confirmed necrotic regions, which manifest naturally as distinct dark regions compared to the autofluorescent, viable tissue. Abcam rat anti-CD68 (1:1000) was used to identify the M1 macrophage profile, while Abcam rabbit anti-mannose receptor (also known as CD206, 1:500) was used to identify the M2 profile. These are established markers of macrophage profile subtypes in inflammation (Didangelos et al., 2014). The antibody for MAC-2 (also known as galectin-3, rat IgG, 1:1000, Cedarlane Labs) was used as a general macrophage marker for cardiac inflammation that is not specific for unique macrophage profiles. Appropriate Alexa Fluor secondary antibodies were used at 1:1000. Detailed immunohistochemistry procedures were performed as reported previously (Gardner and Habecker, 2013).

Analysis

10× (general macrophage response) and 20× (sympathetic innervation and M1/M2 profile) images were used for analysis. The infarct (“I”) encompassed necrotic tissue outlined by fibrinogen staining and was confirmed by loss of autofluorescence using the 350 nm filter. Regions that were approximately one image field removed ($\approx 710 \mu\text{m}$) from the infarct border zone were considered “proximal,” (“P”) whereas regions approximately one more image field farther away from the infarct were considered “distal,” (“D”) (Figure 1A). Three sections collected along different positions of the transverse z-axis were analyzed and averaged from each heart. Sections analyzed from the sham animals were chosen from different regions between the base and the apex.

Sympathetic innervation was quantified using pixel density thresholds generated by ImageJ software as previously described (Gardner et al., 2015). Following analysis of TH innervation density, heart sections for the M1/M2 and MAC2 analysis were chosen based on where the infarct was be maximal. One section from each animal was analyzed at 10× to better encapsulate the entire infarct within one field of view. The infarct perimeter was defined by fibrinogen staining and/or the loss of autofluorescence through the 350 nm filter. Quantification of M1 (CD68), M2 (CD206), and total macrophages (MAC2) was calculated as pixel density thresholds within the infarct perimeter.

Statistics

Prism 5.0 software performed all statistical analysis. Multi-group comparisons across regions and treatments were made using one-way or two-way ANOVA, with subsequent differences between groups using the Tukey’s multiple comparison test (one-way ANOVA) or Bonferroni (two-way ANOVA) *post-hoc* test. Significance was assigned to p-values < 0.05.

Results

To determine if the modulation or loss of PTP σ prevented sympathetic axonal dieback, we carried out I-R surgery to generate MI in PTP $-/-$ mice or WT mice treated with ISP. Sympathetic denervation of the infarcted myocardium was nearly absolute by day 3 following MI in the vehicle-treated group, the ISP-treated group, and the PTP σ $-/-$ group (Figure 1B). Normal sympathetic innervation was represented by the sham-operated control (image data not shown; dashed line in Figure 1B graph). The proximal (P) region immediately adjacent to the infarct was also denervated in all three treatment groups, although to a lesser extent than within the infarct. While the ISP-treated mice and the PTP σ $-/-$ mice appeared to show a modest increase in sympathetic innervation in the proximal region, this trend was not statistically significant. Importantly, in regions further from the infarct (D), ISP treatment and PTP σ $-/-$ mice demonstrated increased sympathetic innervation compared to untreated WT mice. These data suggest PTP σ does not mediate axonal dieback in the heart following I-R, but PTP σ does enhance early-phase axonal density in distal regions, suggesting enhanced regeneration.

Because CSPG manipulations can alter macrophage profiles that affect axonal dieback, we sought to see if PTP σ might affect macrophage profiles following I-R in the heart. We identified the deleterious, or “inflammatory” macrophage profile, “M1,” as well as the more beneficial, reparative profile, “M2,” using antibodies specific for CD68 and CD206, respectively. Consistent with our sympathetic innervation data, macrophage profiles were similar between vehicle controls and ISP-treated animals (Figure 1C). The PTP σ $-/-$ hearts, however, did display increased CD206 near the infarct perimeter as evidenced through more yellow co-labeled cells, but this trend did not reach significance. Importantly, all groups demonstrated a similar total macrophage response following MI as evidenced through MAC2 staining. Sham animals had no fluorescent signal from either the general macrophage (MAC2) or the M1/M2 markers (CD68/CD206; data not shown). These data suggest that PTP σ modulation does not significantly alter the early macrophage response within the scar following I-R.

Discussion

Recently we demonstrated that the lack of PTP σ or its inhibition with ISP resulted in sympathetic re-innervation through infarcted scar tissue (Gardner and Habecker, 2013; Gardner et al., 2015); however, the receptor’s role in sympathetic axonal dieback remained unknown. The results here support the conclusion that PTP σ inhibition or elimination does not mitigate sympathetic axonal dieback in the heart. Furthermore, ISP does not significantly affect the early macrophage response.

The higher sympathetic innervation density we observed in the distal regions of the ISP-treated and PTP σ $-/-$ hearts could have resulted from either reduced axonal dieback or from new axonal growth. Given that PTP σ disruption has been linked to enhanced axonal extension in several contexts (Dyck and Karimi-Abdolrezaee, 2015; Gardner and Habecker, 2013; Gardner et al., 2015; Lang et al., 2015), we expect that ISP administration and PTP σ removal promoted axon growth. Such a mechanism would be CSPG-independent because the CSPGs are restricted to the cardiac infarct (Gardner and Habecker, 2013). One possible mechanism is through modulation of nerve growth factor (NGF) signaling. NGF, which promotes sympathetic axonal extension, is increased in the heart following MI, while PTP σ can dephosphorylate and inhibit NGF’s growth-promoting receptor, tropomyosin-related kinase A (trkA) (Faux et al., 2007). Thus the lack of, or inhibition of, PTP σ would increase NGF-induced TrkA signaling, thereby enhancing sympathetic axon growth.

Injury to the heart and spinal cord leads to marked increases in pro-inflammatory M1 polarization within and surrounding lesion sites. In spinal neurons, this M1 profile is neurotoxic and hinders axonal repair. The M2 profile, on the other hand, is normally favored at later time intervals following injury and encourages axonal regeneration (Gensel and Zhang, 2015). In previous studies, digesting CSPGs with ChABC following contusive spinal injury favored the M2 profile and promoted axonal extension and behavioral recovery (Didangelos et al., 2014). It is presumed that this ChABC-mediated M2 polarization is linked to PTP σ , although the role of PTP σ was not investigated. Consistent with previous studies of cardiac inflammation following I-R, there was a dominance of M1 polarization and minimal M2 polarization in both the ISP and vehicle-treated groups within the scar 3

days after injury. Interestingly, the PTP σ $-/-$ hearts trended toward increased M2 polarization within the infarct, but this trend was not statistically significant and the general macrophage response was similar among all groups. In contrast to the axonal dieback in spinal neurons, which was linked to changes in the macrophage response (Gensel and Zhang, 2015), our data suggest that PTP σ and the macrophage response do not contribute to sympathetic axonal dieback in the heart.

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Abbreviations

PTPσ	protein tyrosine phosphatase sigma
ISP	intracellular sigma peptide
CSPGs	chondroitin sulfate proteoglycans
MI	myocardial infarction
WT	wild-type
Veh	vehicle
ChABC	chondroitinase ABC
NGF	nerve growth factor

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Highlights

- PTP σ manipulations do not prevent sympathetic axonal dieback following MI
- PTP σ manipulations increase innervation density away from the scar
- Targeting PTP σ does not alter the macrophage inflammatory response

Figure 1.

PTPσ modulation or deletion does not prevent sympathetic axonal dieback. (A) Diagram of transverse heart regions used for image analysis: “I” = infarct; “P” = proximal; “D” = distal. (B) Sympathetic axon innervation in the infarct (I), proximal (P), and distal (D) regions. The white traces in row “I” define the infarct perimeter visible through fibrinogen staining (red) and subsequent confirmation using the 350 nm filter where loss of autofluorescence indicates non-viable tissue (data not shown). Sympathetic fibers are defined by tyrosine hydroxylase expression (green). Quantification of tyrosine hydroxylase (TH+) is expressed as a % of myocardium area in the image field. Sham % Area of TH+ = $3.037 \pm 0.089\%$. * = $p < 0.01$ ISP vs. Veh; # = $p < 0.05$ PTPσ vs. Veh. Images are 20×; scale bar = 100 μm; n = 4. (C) Macrophage response 3 days after MI. Immunoreactivity identified M1 (CD68, green) and M2 (CD206, red) macrophage profiles, and total macrophages (MAC2). Yellow denotes the presence of both M1 and M2 macrophage profiles. Ratios of CD206 / CD68 immunoreactivity and MAC2 were quantified within the infarct perimeter using pixel density thresholds. The infarct perimeter is outlined in white and was determined by the loss of autofluorescence through the 350 nm filter. Means ± SD; no significant differences were found; $p > 0.05$. Images are 10×; scale bar = 200 μm; n = 4.