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THROMBIN AND PROTEIN C PATHWAY IN PERIPHERAL NERVE SCHWANN CELLS☆

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Abstract—Thrombin and activated protein C (aPC) bound to the endothelial protein C receptor (EPCR) both activate protease-activated receptor 1 (PAR1) generating either harmful or protective signaling respectively. In the present study we examined the localization of PAR-1 and EPCR and thrombin activity in Schwann glial cells of normal and crushed peripheral nerve and in Schwannoma cell lines. In the sciatic crush model nerves were excised 1 h, 1, 4, and 7 days after the injury. Schwannoma cell lines produced high levels of prothrombin which is converted to active thrombin and expressed both EPCR and PAR-1 which co-localized. In the injured sciatic nerve thrombin levels were elevated as early as 1 h after injury, reached their peak 1 day after injury which was significantly higher (24.4 ± 4.1 mU/ml) compared to contralateral uninjured nerves (2.6 ± 7 mU/ml, t-test $p < 0.001$) and declined linearly reaching baseline levels by day 7. EPCR was found to be located at the microvilli of Schwann cells at the node of Ranvier and in cytoplasm surrounding the nucleus. Four days after sciatic injury, EPCR levels increased significantly (57,785 ± 16602AU versus 4790 ± 1294AU in the contralateral uninjured nerves, $p < 0.001$ by t-test) mainly distal to the site of injury, where axon degeneration is followed by proliferation of Schwann cells which are diffusely stained for EPCR. EPCR seems to be located to cytoplasmic component of Schwann cells and not to compact myelin component, and is highly increased following injury.

Key words: EPCR, PAR-1, protein C pathway, Schwannoma, sciatic nerve, sciatic nerve injury.

INTRODUCTION

Peripheral nerve is a cable-like bundle of axons, with high conduction velocities that are being enabled by the myelin sheath. Myelin, a multilamellar membrane that is formed by Schwann cells in the peripheral nervous system (PNS), enwraps the axon in segments that are separated by the nodes of Ranvier. The outermost layer of the Schwann cell extends microvilli that cover the nodes (Poliak and Peles, 2003). Peripheral nerve diseases are common, including Guillain–Barré syndrome a typical inflammatory disease, diabetic peripheral neuropathy, and tumors which mostly originate at the nerve-sheath in the Schwann cells. The peripheral nervous system tends to get injured mainly by stretch, lacerations and compression. As a result of disease, changes in sensory and/or motor conduction occur (Burnett and Zager, 2004). The specific pathogenesis of the conduction disorder is not yet clear. Possible causes may include inflammation and coagulation factors. There is a growing body of evidence that coagulation pathways may play a significant role in the pathogenesis of inflammatory diseases (Ramachandran et al., 2012; Chapman, 2013). Protease-activated receptor 1 (PAR-1) forms the link between coagulation and inflammation in that it mediates many of the pro-inflammatory effects of thrombin (Gurwitz and Cunningham, 1988; Cunningham and Donovan, 1997; Dery et al., 1998; Vergnolle et al., 2003). We have found that PAR-1 is specifically located

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Abbreviations: ALS, Amyotrophic Lateral Sclerosis; aPC, activated protein C; Dvl-2, dishevelled-2; EPCR, endothelial protein C receptor; LTP, long-term potentiation; MPNST, malignant peripheral nerve sheath tumors; NF1, Neurofibromatosis type 1; PAR1, protease-activated receptor 1; pERK, Phosphorylated extracellular signal-related kinase; PN-1, protease nexin-1; PNS, peripheral nervous system; TM, thrombomodulin; TRAP6, PAR1 agonist.

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at the non-compacted Schwann cell myelin microvilli at the nodes of Ranvier in the sciatic nerve (Shavit et al., 2008). PAR-1 is a target for signaling by various proteases and coagulation factors, mainly thrombin, mediate many of their effects through PAR-1 (Adams et al., 2011; van der Poll et al., 2011; Ramachandran et al., 2012; Ben Shimon et al., 2015). In this context, we demonstrated the occurrence of a conduction block upon the application of thrombin and a PAR-1-specific agonist to exposed rat sciatic nerve (Shavit et al., 2008). In addition, Friedmann et al. found a transient threefold increase in thrombin-like activity 1 day after a sciatic nerve injury (Friedmann et al., 1999). Likewise, in nerve crush experiments performed to study synapse reinnervation in mice lacking protease nexin-1 (PN-1), an endogenous inhibitor of thrombin, Lino et al. found a significant delay in synapse reinnervation in PN-1 knock-out animals compared to wild type. This was associated with both a reduced proliferation and an increased apoptosis of Schwann cells (Lino et al., 2007). Finally, Balezina et al. demonstrated a dose-dependent facilitation of thrombin and PAR1 agonist (TRAP6) on regeneration of mouse peripheral nerve after crush. The maximum neurotrophic effect was observed at low concentrations of thrombin and TRAP6 (Balezina et al., 2005).

Anticoagulant activated protein C (aPC) with its receptor the endothelial protein C receptor (EPCR), can also cleave and activate PAR-1. Protein C is activated by low levels of thrombin and the beneficial effects of such levels in the study of Balenzina et al. suggest activation of the aPC-EPCR pathway. In the central nervous system, this specific pathway has shown to be neuroprotective in that it promotes neovascularization and neurogenesis (Thiyagarajan et al., 2008). Similarly, aPC has been shown to prevent mortality in experimental endotoxemia and sepsis through a mechanism based on PAR1 activation, rather than the anticoagulant properties of this protein (van der Poll et al., 2011). Zhong et al. demonstrated that treatment of mutant SOD1-expressing mice with aPC analogs, delivered after disease onset, slowed progression of Amyotrophic Lateral Sclerosis (ALS)-like disease while increasing lifespan and time of the symptomatic phase (Zhong et al., 2009). In a recent study, we found that different concentrations of thrombin affect long-term potentiation (LTP) through different molecular routes converging on PAR-1. High thrombin concentrations slow onset LTP, whereas low concentrations of thrombin promoted LTP through aPC-EPCR-mediated mechanism (Maggio et al., 2013), thus indicating that PAR-1 activation by aPC-EPCR results in signaling which has opposite effects to that induced by thrombin, leading to different outcomes. Several groups have investigated the biased signaling by PAR-1 activated by thrombin or aPC-EPCR. Bae et al. have found that PAR-1 exodomain is unavailable for interaction with coagulation proteases while in the lipid rafts. The binding of either protein C to EPCR or thrombin to the C-terminal hirudin-like sequence of PAR-1 changes the membrane localization and the conformation of PAR-1 to facilitate its recognition and cleavage by these proteases. (Bae et al., 2007a; Bae et al., 2007b, 2008). Soh and Trejo found that aPC activation of PAR1 is mediated by b-arrestin recruitment and activation of the dishevelled-2 (Dvl-2) scaffold in caveolar microdomains and not by G protein signaling as seen with thrombin activation of PAR1 (Soh and Trejo, 2011). Although the peripheral nerves contain both PAR-1 and components of coagulation pathways, the presence of the protein C pathway in the PNS has not been yet addressed. In the present study, we have mapped EPCR in peripheral nervous system and on Schwann cells in normal state and in disease.

EXPERIMENTAL PROCEDURES

Animals

Animal handling as well as all described experiments were performed in accordance and approved by the Institutional Animal Care and Use Committee of The Chaim Sheba Medical Center, Tel HaShomer, Israel, which adheres to the Israeli law on the use of laboratory animals and NIH rules (permits no: 811/13/ANIM, 939/14/ANIM). Eight- week-old male C57BL6 mice were used for the Thrombin activity assay and for western blot analysis. Eight- week-old male Sprague–Dawley SD rats were used for Immunohistological staining (Envigo laboratories, Israel). The use of different species in the same study is justified by the evidence that on the one hand it is easier to homogenize mice nerves because of the thinner endoneurium, while on the other hand staining rat's nerve is more feasible due to its size and to less background staining from the mouse antibody against Caspr. Eight- week-old male PAR-1 knock out (KO), were purchased from Jackson Laboratory (Bar Harbor, ME). PAR1 KO was provided after backcrossing at The Jackson Laboratory for >8 generations on a C57BL6 background. Mice were used for the Thrombin activity assay and for Phosphorylated extracellular signal-related kinase (pERK) detection.

Crush injury

Mice or rats were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg for mice and 16 mg/kg for rats of xylazine injected intraperitoneally and placed on a thermo-regulated heating pad at 37 °C. The left thigh was sterilized with 70% ethanol, a small incision was made in the thigh, and through the connective tissue between the gluteus and the biceps femoris muscles the nerve was exposed. The crush was produced by tightly compressing the sciatic nerve with flattened forceps for 30 s under a binocular operating microscope. After the sciatic nerve crush, the skin was sutured and animals were kept in a closed chamber heated with a lamp until recovery from anesthesia. Animals were sacrificed 1 h, 1 day, 4 days and 7 days after the procedure using pental by I.P administration.

Cell cultures

We used Schwannoma cell lines as a model of the cytoplasmic Schwann cells, where the non-compacted Schwann cell extends microvilli that cover the node of