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A NOVEL HISTOCHEMICAL METHOD FOR THE VISUALIZATION OF THROMBIN ACTIVITY IN THE NERVOUS SYSTEM☆

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Abstract—Although thrombin has an important role in both central and peripheral nerve diseases, characterization of the anatomical distribution of its proteolytic activity has been limited by available methods. This study presents the development, challenges, validation and implementation of a novel histochemical method for visualization of thrombin activity in the nervous system. The method is based on the cleavage of the substrate, Boc-Asp(OBzl)-Pro-Arg-4MβNA by thrombin to liberate free 4-methoxy-2-naphthylamine (4MβNA). In the presence of 5-nitrosalicylaldehyde, free 4MβNA is captured, yielding an insoluble yellow fluorescent precipitate which marks the site of thrombin activity. The sensitivity of the method was determined *in vitro* using known concentrations of thrombin while the specificity was verified using a highly specific thrombin inhibitor. Using this method we determined the spatial distribution of thrombin activity in mouse brain following transient middle cerebral artery occlusion (tMCAo) and in mouse sciatic nerve following crush injury. Fluorescence microscopy revealed well-defined thrombin activity localized to the right ischemic hemisphere in cortical areas and in the striatum compared to negligible thrombin activity contralaterally. The histochemical localization of thrombin activity following tMCAo was in good correlation with the infarct areas per triphenyltetrazolium chloride staining and to thrombin activity measured biochemically in tissue punches (85 ± 35 and 20 ± 3 mU/ml, in the cortical and striatum areas respectively, compared to 7 ± 2 and 13 ± 2 mU/ml, in the corresponding contralateral areas; mean ± SE; $p < 0.05$). In addition, 24 h following crush injury, focal areas of highly elevated thrombin activity were detected in teased sciatic fibers. This observation was supported by the biochemical assay and western blot technique. The histochemical method developed in this study can serve as an important tool for studying the role of thrombin in physiological and pathological conditions. **© 2016 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: ischemic stroke, thrombin, transient middle cerebral artery occlusion, crushed sciatic nerve, enzyme histochemistry.

INTRODUCTION

Thrombin has, in addition to its role in thrombogenesis, important hormone-like activities that affect various cells in the brain through the activation of its receptor, the protease-activated receptor 1 (PAR1) (Bar-Shavit et al., 1986; Coughlin, 2000; Junge et al., 2003). Activation of this receptor by low concentrations of thrombin may have neuroprotective effects while at higher concentrations thrombin has deleterious effects (Xue and Del Bigio, 2001; Noorbakhsh et al., 2003; Xi et al., 2003; Chen et al., 2010; Kameda et al., 2012). Previously, using a novel and specific biochemical method for direct quantitative detection of thrombin activity in brain slices, we have found that following both permanent (Bushi et al., 2013) and transient (Bushi et al., 2015) middle cerebral artery occlusion (MCAo) performed in mice, thrombin activity is elevated throughout the ischemic hemisphere reaching peak levels at the ischemic core. Spatial distribution analysis indicates that following transient MCAo transient middle cerebral artery occlusion (tMCAo) thrombin activity is elevated in both the infarct area and peri-infarct areas (Bushi et al., 2015). These results are in agreement with the study performed by Chen et al. showing that during acute ischemic stroke, disruption of the blood–brain

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Abbreviations: 4MβNA, 4-methoxy-2-naphthylamine; BBB, blood–brain barrier; CNS, central nervous system; MCAo, middle cerebral artery occlusion; mTBI, minimal traumatic brain injury; NAPAP, Nα-(2-Naphthylsulfonylglycyl)-4-Amidino-(D,L)-Phenylalanine Piperidide Acetate; NSA, 5-nitrosalicylaldehyde; OGD, oxygen glucose deprivation; PAR1, proteases- activated receptor 1; PBS, phosphate- buffered saline; tMCAo, transient middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride.

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barrier (BBB) allows higher concentrations of blood-derived thrombin to enter the brain (Chen et al., 2012). Moreover, several *in vitro* studies have shown that thrombin is synthesized in the brain upon oxygen glucose deprivation (OGD) simulating ischemia (Thevenet et al., 2009; Stein et al., 2015). In this context, thrombin has been shown to cause synaptic dysfunction (Maggio et al., 2008; Maggio et al., 2013a,b; Becker et al., 2014; Prabhakaran et al., 2015) and later on neuronal damage (Junge et al., 2003; Suo et al., 2004; Hamill et al., 2009), through the activation of PAR1 (Chen et al., 2012). Indeed, the toxic effects of thrombin in the central nervous system (CNS) have been shown in various ischemic models, inflammatory and neurodegenerative brain diseases (Yin et al., 2010; Rami, 2012; Chapman, 2013; Davalos et al., 2014), where neuroprotection could be achieved either by PAR1 deletion as shown by the group of Traynelis (Junge et al., 2003; Hamill et al., 2009) or with thrombin inhibitors (McColl et al., 2004; Chen et al., 2012; Lyden et al., 2014).

In addition to the CNS, there are many lines of evidence suggesting that thrombin and its receptors have important roles in peripheral nerve diseases. Significant increase in thrombin-like activity was observed in sciatic nerves that were taken from rat and mice 1 and 2 days respectively after crush injury (Smirnova et al., 1996; Friedmann et al., 1999). Moreover, we have described the localization of PAR1 at the nodes of ranvier and functionally activation of these receptors results in conduction block (Shavit et al., 2008).

In contrast to immunohistochemical methods that localize the enzyme protein whether it is active or not, histochemical visualization of the activity of an enzyme is a powerful approach to study whether an enzyme is functionally involved in a pathophysiological process because it links the enzyme activity to cell and tissue structure. Although thrombin has an important role in both central and peripheral nerve diseases, characterization of the anatomical distribution of its proteolytic activity in the nervous system has been limited by available methods. With the rapid growth in the field of thrombin-based therapy it is becoming increasingly clear that the histochemical study of thrombin activity will be important in understanding the mechanisms that regulate its function during both central and peripheral nerve diseases. Recently, Chen et al. elegantly detected the location of thrombin activity in rat brains following ischemic stroke using a novel cell-penetrating peptide-imaging probe that was infused into the blood stream and then entered brain tissue through vascular disruption (Chen et al., 2012). Our study presents a new alternative method to visualize the sites of thrombin activity in all brain regions and it is not dependent on reperfusion as in the methodology of Chen et al. Moreover, this fluorescent histochemical method is relatively simple, uses commercially available products, and can be performed ex vivo with different tissue types. The method is shown to demonstrate the distribution of thrombin activity in mice brain following tMCAo and in mice sciatic nerve following crush injury.

EXPERIMENTAL PROCEDURES

The histochemical method is based on the landmark work of Dolbeare and Smith (1977). In the early stage of the development we used the commercial thrombin substrate Z-Gly-Pro-Arg-4MβNA (J-1120, Bachem, Switzerland). However, as described in the results section, we found that the specificity and sensitivity of this substrate does not fulfill the requirements for an appropriate histochemical method to localize thrombin activity. Thus, the substrate that we finally used is Boc-Asp(OBzl)-Pro-Arg-4MβNA, a thrombin substrate that was specially synthesized per our request (GL Biochem, Shanghai, China), containing the sequence Asp(OBzl)-Pro-Arg that was found to be one of the most sensitive sequences for cleavage by thrombin (Kawabata et al., 1988).

4-methoxy-β-naphthylamine (4MβNA) is a known soluble fluorophore that produces a blue fluorescence (emission = 425 nm [nm]) following excitation by wavelength of 340 [nm]. However, when 4MβNA interacts with 5-nitrosalicylaldehyde (NSA) it produces a schiff-base complex with a shift in fluorescence from yellow to orange which is water insoluble and potentially trapped in the tissue (Dolbeare and Smith, 1977; Smith, 1983; Back and Gorenstein, 1989; Cataldo and Nixon, 1990; Rudolphus et al., 1992; Kamiya et al., 1998) (Fig. 1). In our previous work we have found that thrombin activity is raised dramatically in the ischemic hemisphere 24 h following both permanent (Bushi et al., 2013) and transient (Bushi et al., 2015) MCAo. In addition, thrombin is elevated in sciatic nerves following crush injury (Smirnova et al., 1996; Friedmann et al., 1999). Thus, we assumed that thrombin that is generated in the ischemic tissue and in crushed nerve will potentially cleave the thrombin substrate Boc-Asp(OBzl)-Pro-Arg-4MβNA releasing free 4MβNA that can react with NSA to yield insoluble 4MβNA–NSA yellow fluorescent complexes marking the site of thrombin activity (Fig. 1).

Optimized substrate concentration

Since in this study we used for the first time the custom-made thrombin substrate Boc-Asp(OBzl)-Pro-Arg-4MβNA, our first step was to determine its most effective concentration. We determined this by comparing the cleavage rates of different substrate concentrations by 50 mU/ml of bovine thrombin (Sigma–Aldrich, Israel). Different concentrations of Boc-Asp(OBzl)-Pro-Arg-4MβNA substrate (0.01, 0.05, 0.1, 0.4, 0.8 mM) were added to a 96-well black microplate (Nunc, Denmark), each well in the microplate contained thrombin buffer (50 mM TRIS/HCl, pH 8.0, 0.15M NaCL, 1 mM $CaCl_2$), 0.1% BSA and 50 mU/ml of bovine thrombin. Thrombin cleaved the substrate and released free 4MβNA that was measured by a fluorescence reader (Molecular Devices, USA) with excitation and emission filters of 340 and 425 nm respectively. Cleavage rate was measured by the linear slope of the fluorescence intensity vs. time. As shown in the results section, the optimal substrate concentration was found to be 0.1 mM.

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