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Enzyme specificity and effects of gyroxin, a serine protease from the venom of the South American rattlesnake *Crotalus durissus terrificus*, on protease-activated receptors

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ABSTRACT

Gyroxin is a serine protease displaying a thrombin-like activity found in the venom of the South American rattlesnake *Crotalus durissus terrificus*. Typically, intravenous injection of purified gyroxin induces a barrel rotation syndrome in mice. The serine protease thrombin activates platelets aggregation by cleaving and releasing a tethered N-terminus peptide from the G-protein-coupled receptors, known as protease-activated receptors (PARs). Gyroxin also presents pro-coagulant activity suggested to be dependent of PARs activation. In the present work, the effects of these serine proteases, namely gyroxin and thrombin, on PARs were comparatively studied by characterizing the hydrolytic specificity and kinetics using PARs-mimetic FRET peptides. We show for the first time that the *short* (*sh*) and *long* (*lg*) peptides mimetizing the PAR-1, -2, -3, and -4 activation sites are all hydrolyzed by gyroxin exclusively after the Arg residues. Thrombin also hydrolyzes PAR-1 and -4 after the Arg residue, but hydrolyzes *sh* and *lg* PAR-3 after the Lys residue. The k_{cat}/K_M values determined for gyroxin using *sh* and *lg* PAR-4 mimetic peptides were at least 2150 and 400 times smaller than those determined for thrombin, respectively. For the *sh* and *lg* PAR-2 mimetic peptides the k_{cat}/K_M values determined for gyroxin were at least 6500 and 2919 times smaller than those determined for trypsin, respectively. The k_{cat}/K_M values for gyroxin using the PAR-1 and -3 mimetic peptides could not be determined due to the extreme low hydrolysis velocity. Moreover, the functional studies of the effects of gyroxin on PARs were conducted in living cells using cultured astrocytes, which express all PARs. Despite the ability to cleavage the PAR-1, -2, -3, and -4 peptides, gyroxin was unable to activate the PARs expressed in astrocytes as determined by evaluating the cytosolic calcium mobilization. On the other hand, we also showed that gyroxin is able to interfere with the activation of PAR-1 by thrombin or by synthetic PAR-1 agonist in cultured astrocytes. Taken together, the data presented here allow us showing that gyroxin cleaves

Abbreviations: PARs, protease-activated receptors; FRET, Fluorescence Resonance Energy Transfer; GPCRs, G-protein-coupled receptors; BBB, blood brain barrier; *sh*, short; *lg*, long; CNS, central nervous system; RSDs, retinal spreading depression waves.

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PARs-mimetic peptides slowly and it does not induce activation of PARs in astrocytes. Although gyroxin does not mobilize calcium it was shown to interfere with PARs activation by thrombin and PAR-1 agonist. The determination of gyroxin enzymatic specificity and kinetics on PAR-1, -2, -3, and -4 will potentially help to fill the gap in the knowledge in this field, as the PARs are still believed to have a key role for the gyroxin biological effects.

1. Introduction

Gyroxin is a serine protease that accounts for about 2% of the total protein content of the crude venom of the South American rattlesnake *Crotalus durissus terrificus*. The intravenous injection of this thrombin-like protease into mice triggers the barrel rotation syndrome, which is characterized by rotations of the animal around its long axis (Barrio, 1961). Although the suggestion of a putative intracerebral action of gyroxin involving neurotransmitters release (Cohn and Cohn, 1975), it was shown by others that gyroxin does not affect the release of dopamine and acetylcholine *in vitro* (Camillo et al., 2001).

Several studies exploring different aspects of gyroxin features were reported in the recent years including: (1) the existence of gyroxin structural isoforms, demonstrated by the cloning of five different sequences from a cDNA library of *C. d. terrificus* venom glands (Yonamine et al., 2009); (2) the gyroxin ability to increase blood brain barrier (BBB) permeability (Alves da Silva et al., 2011); (3) the gyroxin action in central nervous system (CNS) suggested by its ability to modify the optical profiles of retinal spreading depression waves (RSDs) in chick retina (Da Silva et al., 2012); and (4) the gyroxin triggered activation of platelet aggregation, potentially mediated by protease-activated receptors (PARs) activation (Da Silva et al., 2012), suggested based on the thrombin mechanism of action that requires PARs to trigger the platelet aggregation (Coughlin, 1999; Kahn et al., 1998).

PARs are members of seven-transmembrane G-protein-coupled receptors (GPCRs) family, whose activation is triggered by the cleavage of specific sites at the N-terminus of receptor by serine proteases, resulting in the generation of a tethered ligand that interacts with the receptor within its extracellular loop-2 (Macfarlane et al., 2001). The binding of this ligand to the core of PARs initiates an intracellular signal transduction pathway, which involves both phosphoinositide breakdown and cytosolic calcium mobilization (Macfarlane et al., 2001).

The mechanism of activation of PARs (-1, -2, -3, and -4) by serine proteases followed by the intracellular signaling pathways, and their consequent physiological and pathophysiological roles are well studied (Macfarlane et al., 2001). Rat astrocytes express PAR-1, -2, -3, and -4, but the calcium signal evoked by PAR-3 and -4 agonist peptides is relatively weaker compared to that induced by PAR-1 or -2 agonists (Wang et al., 2002).

Activation of human platelets by thrombin involves the proteolytic activation of PAR-1 and PAR-4 (Coughlin, 1999; Kahn et al., 1998). Low concentrations of thrombin is enough to activate PAR-1 in human platelets, due to its high-affinity, while PAR-4 is a low-affinity receptor, requiring higher concentrations of thrombin for signaling activation (Mao et al., 2008).

Gyroxin also promotes platelet aggregation and the involvement of PARs was suggested (Da Silva et al., 2012). In fact, a significant inhibition of platelet aggregation induced by gyroxin was observed in the presence of antagonists of PAR-1 [SCH79797] or PAR-4 [tcY-NH2]. Interestingly, PAR-1 antagonist inhibits platelet aggregation triggered by gyroxin at concentrations of about two orders of magnitude smaller than that required for PAR-4 antagonist, and the combination of these two antagonists determined only a partial inhibition of the platelet aggregation induced by gyroxin (Da Silva et al., 2012).

Up to now, despite the several similar enzymatic features shared by both serine proteases, *e.g.* gyroxin and thrombin, the potential effects of gyroxin on PARs are still poorly described. So forth, the characterization of the enzymatic specificity of gyroxin on PAR-1, -2, -3, and -4, and the evaluation of the PARs activation by gyroxin in living cells, monitored by the free intracellular calcium concentration ($[Ca^{2+}]i$) increases might help to fill the gap in the knowledge in this field.

2. Material and methods

Ethics statement: The protocol was approved by the Committee on the Ethics of Animal Experiments of Universidade Federal de Sao Paulo (UNIFESP) (CEP Number: 2003/09).

2.1. Purification of gyroxin

Crotamine negative venom of *C. d. terrificus* was kindly provided by Dr. Eduardo B. Oliveira (Departamento de Bioquímica e Imunologia, Universidade de São Paulo, Ribeirão Preto, Brazil). Gyroxin was obtained by fractionating the crude venom by employing affinity chromatography and molecular exclusion as previously described (Alves da Silva et al., 2011). As gyroxin is a serine protease, the use of organic solvent was consistently avoided to assure that the enzymatic activity properties were not lost in the purification steps. The collected fractions containing purified gyroxin were combined in a conical tube, and the protein concentration was determined by Bradford method (Bradford, 1976). The samples were cooled, lyophilized and stored at −20 °C.

2.2. Neurotoxicity assay

To confirm that the biological activity of gyroxin was not lost during the purification process, a neurotoxicity assay that allows observing the barrel rotation syndrome