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Effects of morin on snake venom phospholipase A_2 (PLA_2)

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Abstract

Flavonoids are potent anti-inflammatory compounds isolated from several plant extracts, and have been used experimentally against inflammatory processes. In this work, a PLA_2 isolated from the *Crotalus durissus cascavella* venom and rat paw oedema were used as a model to study the effect of flavonoids on PLA_2. We observed that a treatment of PLA_2 with morin induces several modifications in the aromatic amino acids, with accompanying changes in its amino acid composition. In addition, results from circular dichroism spectroscopy and UV scanning revealed important structural modifications. Concomitantly, a considerable decrease in the enzymatic and antibacterial activities was observed, even though anti-inflammatory and neurotoxic activities were not affected. These apparent controversial results may be an indication that PLA_2 possess a second pharmacological site which does not affect or depend on the enzymatic activity.

Keywords: PLA_2; Circular dichroism spectroscopy; Antibacterial activity; Flavonoids; Morin; *Crotalus durissus cascavella*; Neurotoxic

1. Introduction

Phospholipase A_2 (PLA_2) present in mammal cells exhibit different modes of action. Evidence in this direction has been reported, revealing differences in activity, function and interaction among different PLA_2s in the regulation of the arachidonic acid (AA) metabolism and phospholipids turnover, in addition to the control of inflammatory processes involving AA metabolism (Murakami et al., 1998). Accordingly, the inflammatory events evoked by PLA_2s are primarily associated with the enzymatic activity and release of AA metabolites. However, catalytically inactive Lys49 PLA_2s trigger inflammatory and nociceptive responses comparable to those of their catalytically active counterparts, thereby evidencing that these proteins promote inflammation and pain by mechanisms not related to phospholipid hydrolysis nor to mobilization of arachidonic acid (Teixeira et al., 2003). Also, there are several reports of snake venom PLA_2 inducing oedema, an effect that, in some cases, is dependent on the ability of the PLA_2 to bind to specific membrane proteins. Different structure-function studies have been carried out, indicating that the CRD5 domain of the M-type receptor is the key element in $sPLA_2$

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binding. Residues near or within the Ca^{2+} loop of $sPLA_2$ are involved in the binding of the enzyme to M-type receptors; binding of $sPLA_2$ to the receptor leads to an inhibition of its catalytic activity (Valentin and Lambeau, 2000). Further support to this view is the mode of action of *p*-bromophenacyl bromide (*p*-BPB), that covalently bind to His (48) in the PLA_2 catalytic site, inducing the lost of enzymatic activity and a significative decrease of the pharmacological effect, whether in the enzymatically active or inactive enzyme (Zhao et al., 1988; Chandra et al., 2002). Crystallographic studies carried out by Zhao and colaborators (Zhao et al., 1998) showed that *p*-BPB modifies the hydrophobic channel profile, inducing slight conformational changes in the Ca^{2+}-binding loop but significant structural changes elsewhere in the molecule.

Flavonoids are a group of naturally occurring polyphenolic compounds widely distributed in the plant kingdom. Some flavonoids have been found to have interesting medicinal properties, exerting antilipoperoxidant, anti-inflammatory, antiallergic, antiviral, antibacterial and anticancer effects (Middleton and Drzewiecki, 1984; Harborne, 1988; Di Carlo et al., 1999). In spite of the fact that flavonoids exhibit an antioxidant action (Bors et al., 1990), some flavonoids have been found to exhibit pro-oxidant effects in vitro (Hodnick et al., 1994; Cao et al., 1995), a property which have been attributed to the fact that flavonoids can undergo autoxidation in aqueous solutions in the presence of transition metals, leading to the formation of highly reactive $^{\cdot}OH$ radicals (Rice-Evans et al., 1996; Cao et al., 1997). Recently reported studies using various plant species have shown that flavonoids induce a dose-dependent inhibition of in vitro phospholipid hydrolysis in both secretory and cytossolic PLA_2s (Fawzy et al., 1988; Vishwanath et al., 1988). Flavonoids also exhibit different inhibitory levels for group I PLA_2 from porcine pancreas and *Naja naja*, and for group II PLA_2 from *Vipera russeli* and *Crotalus atrox*. The most important regions involved in the inhibition of PLA_2 were reported to be hydroxyl groups in 3′- and 4′-positions (Lindahl and Tagesson, 1997; Rotelli et al., 2003). Group II enzymes have been found at inflammatory sites in animal models, as well as in synovial fluids from patients with rheumatoid arthritis and various human inflammatory disease states, where a correlation between serum PLA_2 levels and disease activity is observed. Moreover, exogenous administration of secretory PLA_2, such as snake venom PLA_2, can induce or exacerbate inflammatory response in animals (Gil et al., 1997; Toyama et al., 2003).

Flavonoids might be involved in the inhibition of several enzymes; however, the precise modulation or the modes of action of these compounds are not clear. In particular, morin has been reported to exhibit beneficial biological effects in vitro, with a strong anti-inflammatory activity, reducing the intensity of experimental septic shock by decreasing macrophage activity; it may also regulate immune response by modulating cytokine profiles (Fang et al., 2003). To better understand the mechanism of action of morin on isolated PLA_2 and its implications for the structure and biological activities of snake venom PLA_2, a variety of techniques have been used in the present work. Several amino acid modifications are induced when treating PLA_2 with morin. Considerable differences on the activities and secondary structure of PLA2 were also observed. These results were discussed on the light of pharmacological and biochemical data available.

2. Material and methods

2.1. Venoms and reagents

Venom from *Crotalus durissus cascavella* was kindly donated by the Instituto Butantan (São Paulo, Brazil). Morin was obtained from Sigma Co., Ltd (USA). Solvents, chemicals and reagents used in protein purification and characterization of HPLC grade or higher were acquired from Sigma-Aldrich chemicals (USA), Merk (USA) and Bio-Rad (USA). Male and Female Wistar rats (120–150 g) and Swiss mice (18–20 g) used in the pharmacological assays were obtained from the University's Central Animal House. All animal experiments were approved by the State University of Campinas Ethics Committee (São Paulo, Brazil).

2.2. Purification of PLA_2

C. d. cascavella whole venom (45 mg) was completely dissolved in 0.2 M ammonium bicarbonate buffer (pH 7.9) until complete homogenization followed by a clarification step using a high-speed centrifugation ($4500 \times g$ for 2 min). The supernatant was recovered and applied on a HPLC molecular exclusion chromatography column (1×60 cm, Pharmacia) previously equilibrated with the same buffer used for venom dissolution. The main fractions of the venom were purified at a constant flow rate of 0.3 ml/min. The chromatographic run was monitored at A_{280} nm and then the crotoxin-like protein was eluted, pooled and lyophilized for further reverse phase HPLC. The whole crotoxin was subjected to a second chromatographic step using a reverse phase HPLC. This crotoxin was dissolved in 200 μl of TFA (triflouroacetic acid; 0.1%; buffer A) until complete dissolution, followed by clarification using a high-speed centrifugation ($4500 \times g$ for 3 min). The supernatant was then loaded on a μ-Bondapack C18 reverse phase HPLC column (0.78×30 cm). Protein elution was done using a non-linear gradient of buffer B (66.6% of acetonitrile in TFA 0.1%) at a constant flow rate of 2.0 ml/min. The chromatographic run was monitored at A_{214} nm and the fraction obtained was then lyophilized. The level of the purity of the isolated PLA_2 was evaluated by Tricine SDS-PAGE according to Shagger and von Jagow (1987).