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Anticoagulant effect of *Naja naja* venom 5′nucleotidase: Demonstration through the use of novel specific inhibitor, vanillic acid

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Abstract

The snake venom proteins affect hemostasis by either advancing/delaying blood coagulation. Apart from proteases and phospholipase A_2s (PLA_2s), 5′nucleotidase is known to affect hemostasis by inhibiting platelet aggregation. In this study, the possible involvement of *Naja naja* venom 5′nucleotidase in mediating anticoagulant affect is evaluated. Vanillic acid selectively and specifically inhibited 5′nucleotidase activity among other enzymes present in *N. naja* venom. It is a competitive inhibitor as evident of inhibition relieving upon increased substrate concentration. Vanillic acid dose dependently inhibited the anticoagulant effect of *N. naja* venom up to 40%. This partial involvement of 5′nucleotidase in mediating anticoagulant effect is substantiated by concanavalin-A (Con-A) inhibition studies. Con-A, competitively inhibited in vitro protease and 5′nucleotidase activity up to 100%. However, it did not exhibit inhibitory activity on PLA_2. The complete inhibition of anticoagulant effect by Con-A upon recalcification time suggests the participation of both 5′nucleotidase and protease in mediating anticoagulant effect of *N. naja* venom. Vanillic acid and Con-A inhibition studies together suggest that probably 5′nucleotidase interacts with one or more factors of intrinsic pathway of blood coagulation to bring about anticoagulant effect. Thus, this study for the first time demonstrates the involvement of 5′nucleotidase in mediating *N. naja* venom anticoagulant effect.

Keywords: 5′nucleotidase; *Naja naja*; Vanillic acid; Anticoagulant; Hemostasis

1. Introduction

Components acting on hemostasis are generally found in snake venoms belonging to viperidae, crotalidae and elapidae families. Interference with blood coagulation is one of the main causes of pathological manifestations exhibited by bite of snakes belonging to the families, crotalidae and viperidae. Even though such manifestations are not easily observed in elapidae snakebite, few reports show the involvement of venom components on blood coagulation system when studied in vitro (Lee et al., 1995; White, 2004 and references therein). A recent in vitro study has shown that phospholipase

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A_2 (PLA_2) enzyme and prothrombin activator from *Naja naja* venom have shown effects on platelets and blood clotting factors (Sundell et al., 2003). A number of snake venom toxins that affect blood coagulation as procoagulant or anticoagulant have been purified and characterized (Hutton and Warrell, 1993; Markland, 1998; Kini et al., 2002).

The anticoagulant effects of venoms have been mainly attributed to proteases and PLA_2s (Markland, 1997; Kini, 2005). 5′nucleotidase an ubiquitous enzyme of snake venoms has been shown to act as a co-factor of hemorrhagic toxins and also known to affect hemostasis by modulating platelet functions (Dimitrov and Kankonkar, 1968; Aird, 2002, Kini and Evans, 1990). Boffa and Boffa (1974) demonstrated 5′nucleotidase as the most potent inhibitor of platelet aggregation from *Vipera aspis*. The 5′nucleotidase purified from *Trimeresurus graminus* is known to inhibit platelet aggregation induced by ADP, sodium arachidonate, collagen, ionophore A-23187 and thrombin (Ouyang and Huang, 1983). This inhibition of platelet aggregation by 5′nucleotidase is known to consequently suppress blood coagulation without platelet lysis. Thus it is now hypothesized that 5′nucleotidase probably acts synergistically with other toxins such as ADPases, phospholipases and disintegrins to exert more pronounced anticoagulant effect (daSilva and Aird, 2001). Although it is widely accepted that 5′nucleotidase alters platelet function (Kini and Evans, 1990; Kini and Chow, 2001), its action on blood coagulation cascade remains unknown, indicating that the enzyme has not been well characterized in snake venoms.

Numerous studies have used specific inhibitors of enzymes as biochemical and pharmacological tool for their characterization (Lazarovici and Lelkes, 1992; Ferry et al., 2004; Erion et al., 2005). Several snake venom toxins/enzymes were inhibited by components isolated from traditional folk medicinal plants used against snakebite (Okonogi et al., 1979; Pithayanakul et al., 2004; Soares et al., 2005). It has been observed that a striking parallelism exists between the capability of plants and their chemical compounds in neutralizing the action of snake venom toxicity and anti-inflammatory properties (Mors et al., 2000). With these inhibitors, mechanism of action of several venom toxins/enzymes has been elucidated (Vishwanath et al., 1987; Cirino et al., 1989; Jayaraman et al., 1999; Mors et al., 2000). Vanillic acid (4-hydroxy-3-methoxy benzoic acid) is a phenolic derivative known to possess antimicrobial (Delaquis et al., 2005), anti-filarial (Varma et al., 1993) and hepatoprotective activities (Singh et al., 2005). Further, it has been shown that it is one of the main components of a traditional Chinese folk medicine formulation—Di-Gu-Pi decoction, prepared from the root bark of *Lycium barbarum* L. This extract is used for the treatment of diabetes, hemorrhagic inflammation, hypertension, ulcers, and fever in traditional Chinese medicine (Li et al., 2004). Vanillic acid a major chemical constituent of vanilla, a neutraceutical plant, inhibited specifically 5′nucleotidase activity and not proteases and PLA_2 that are commonly involved in hemostasis. The other pharmacological effect that is neutralized by vanillic acid is anticoagulant activity of *N. naja* venom. This study clearly demonstrates the involvement of *N. naja* venom 5′nucleotidase, a glycoprotein in exerting anticoagulant effect using novel non-nucleoside specific inhibitor—vanillic acid.

2. Materials and methods

2.1. Materials

Cobra (*N. naja*) venom was purchased from Haffkins Institute, Mumbai, India. *Escherichia coli* (lyophilized cells of strain W (ATCC 9637) and oleic acid (C^{14}) was obtained from Perkin Elmer Life Sciences Inc., Boston, MA, USA. Fat-free bovine serum albumin (BSA) fraction V was purchased from PAA Laboratories GmbH Haidmannweg, Austria. Con-A (concanavalin-A type V), casein and human fibrinogen were purchased from Sigma Chemical Company St. Louis, MO, USA. Adenosine 5′monophosphate (5′AMP), α-methly-D-glucoside, α-methyl-D-mannoside and vanillic acid were purchased from Sisco Research Laboratories, Bangalore, India. All other chemicals and reagents purchased were of analytical grade. Fresh human blood samples were collected from healthy volunteers of Department of Biochemistry, University of Mysore, India. All the assays were done using double distilled water. For all the studies, venom and inhibitors were dissolved in saline.

2.2. Protein estimation

Protein concentration was determined according to the method of Lowry et al. (1951) using BSA as standard.