Analysis of nociceptive effects of neurotoxic phospholipase A2 from *Vipera nikolskii* venom in mice

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ABSTRACT

Phospholipases A2 are represented in snake venoms by several types and possess diverse biological activities including neurotoxicity. Previously, we isolated and characterized two neurotoxic phospholipases A2 (HDP-1 and HDP-2) from the venom of Nikolski's viper (*Vipera nikolskii*), which were heterodimers composed of two non-covalently bound subunits. Each heterodimer consisted of an enzymatically active basic subunit and an inactive acidic subunit. In this work, we studied the in vivo biological activity of HDP-2 in mice. The acute toxicity (LD$_{50}$ = 0.38 μg/gm) and maximal tolerated dose (0.1 μg/gm) were determined. In the hot plate test, HDP-2 at the maximal tolerated dose, reliably prolonged the time of the mouse staying on the plate. However, taking into account the neurotoxicity of HDP-2, we believe that this effect may be explained by a general intoxication rather than specific decrease of pain sensitivity. In this respect HDP-2 differs from other heterodimeric phospholipases A2 like crotoxin, which possess analgesic activity. This difference can be explained by the dissimilarity in the structure of the acidic subunits, suggesting an important role of this subunit in analgesic activity.

KEYWORDS: Phospholipase A2, toxicity, venom, snake, nociception, α-neurotoxin

INTRODUCTION

One of the most prominent early symptoms of snakebite envenoming is disturbed pain sensitivity. Snakebites often induce severe pain at the site of the bite or in other parts of the body (Frangides et al, 2006; Alkaabi et al, 2011; Walker and Morrison, 2011). This is especially pronounced in bites by snakes of the family Viperidae. However, another set of data indicates anti-nociceptive properties of some snake venoms (Picolo et al, 1998) and their components. Thus, analgesic activity has been shown for so-called three-fingered α-neurotoxins. For example, α-cobratoxin from the Thai monocelate cobra *Naja kaouthia* (Chen et al, 1998) and their components. Thus, analgesic activity has been shown for so-called three-fingered α-neurotoxins. For example, α-cobratoxin from the Thai monocelate cobra *Naja kaouthia* (Chen et al, 1998) produced antinociceptive effects. Recently, it has been shown that a new class of three-finger peptides from the black mamba (*Dendroaspis polylepis*) venom is able to abolish pain through inhibition of acid sensing ion channels expressed either in central or peripheral neurons (Diochot et al, 2012). Antinociceptive activity was also reported for crotoxin, a phospholipase A2 (PLA2) from South American rattlesnake (*Crotalus durissus terrificus*) venom (Zhang et al, 2006). The crotoxin molecule is composed of two non-covalently bound subunits: a weakly toxic basic phospholipase A2 and an acidic non-toxic and non-enzymatic polypeptide named crotapotin. Similar to some other oligomeric PLA2, it possesses presynaptic neurotoxicity (Sampaio et al, 2010). In our studies of Nikolski's viper (*Vipera nikolskii*) venom, we isolated two heterodimeric PLA2 (HDP-1 and HDP-2) also manifesting presynaptic neurotoxicity (Ramazanova et al, 2008). Crotoxin and *V. nikolskii* PLA2s are homologous proteins; the main structural difference between them is in their acidic subunits. The acidic crotapotin consists of three disulfide-linked polypeptide chains (α, β, γ) which result from proteolytic
cleavage of a unique precursor (pro-CA) that has been identified from its cDNA (Bouchier et al, 1991). In HDP-1 and HDP-2 the acidic subunit consists of a single polypeptide chain that is homologous to the basic subunit, but lacks a histidine residue at the active site (Ramazanova et al, 2008). Such dissimilarity may result in different biological activities of proteins. The present work was undertaken to investigate whether neurotoxic HDP-2 has analgesic properties. We found that HDP-2 increases the time before the first hind paw licking and the first jump of CD-1 mice in the hot plate test. This might suggest decreased pain sensitivity. However, as intoxication by HDP-2 strongly inhibits locomotor activity in mice, this effect may be explained by a general neurotoxic effect of the toxin.

MATERIALS AND METHODS

The phospholipase HDP-2 was isolated from V. nikolskii venom as described (Ramazanova et al, 2008). For injection in mice, the protein was dissolved in saline. Adult male mice of the CD-1 strain (8-9 weeks old, 30-35 gm body weight) were used in this study. The animals were kept in a 12 hr light:dark cycle (18-26°C, 30-70% humidity) with food and water ad libitum in accordance with the World Health Organization’s International Guiding Principles for Animal Research (WHO Chronicle, 1985). All animals were subjected to experimental operations only once and were not used for other tests. Intravenous injection of a single toxin dose was used for toxicity assays. The injection volume was 1 ml per 1000 gm of animal body weight. Animals were observed for 72 hr after injection. The quantitative toxicity parameters were calculated by the method of Litchfield and Wilkoxon (1949).

The hot plate test was performed on a Hot Plate Analgesia Meter (Columbus Instruments, Columbus, OH, USA). The animals were injected with HDP-2 (0.1 mg/kg) and placed on the thermostat surface at 55°C 15 min after injection. Latency to paw-lick response and latency to jumps was registered. Animals were taken off the hot surface after the first jump. Sodium chloride solution (0.9%) was injected into a control group of animals.

RESULTS AND DISCUSSION

Previously, we isolated two heterodimeric phospholipases A2 (HDP-1 and HDP-2) from V. nikolskii venom and showed their neurotoxic effects in vitro (Ramazanova et al, 2008). In particular, the nerve impulse transmission in the frog nerve-muscle preparation was affected. HDP-1 and HDP-2 are structurally and functionally very similar. However HDP-2 possesses higher biological activity and its content in the venom is also higher, that is why HDP-2 was chosen for this study. It was isolated from the V. nikolskii venom by ion-exchange chromatography as described (Ramazanova et al, 2008). To further study the biological activity in vivo, the mice were injected intravenously with increasing doses of HDP-2. The symptoms of HDP-2 intoxication were similar irrespective of the dose used. One to 3 min after injection, the animals showed severe depression, a strong decrease of locomotor activity, decreased breath rate and crooked posture followed at high doses by coma within the next 15-30 min. Their death was registered within the first 2 hrs. The general conditions of surviving animals were normal. The results of postmortem investigations showed that death was caused by asphyxia as manifested by wide eyes, a protruding tongue as well as cyanosis of the lips and the extremities. There were no obvious changes of internal organs. The data of the toxicity assays are given in Table 1. The calculated LD₅₀ of HDP-2 is 0.38 μg/gm. This value is close to that of a heterodimeric PLA₂ from Vipera aspis venom (0.288 μg/gm; Komori et al, 1990) and about two times higher than the value determined for a heterodimeric PLA₂ from Taiwanese Daboia siamensis (Wang et al, 1992). The LD₅₀ for crotoxin is 0.06-0.09 μg/gm (Okamoto et al, 1993; Rangel-Santos et al, 2004), therefore the toxicity of HDP-2 was substantially lower than that of crotoxin. The maximal tolerated dose of HDP-2 was 0.1 μg/gm. This value was used for the further study of HDP-2 influence on pain sensitivity.

Table 1. The lethality of HDP-2 after single intravenous injection in mice.

| Number of mice injected | Dose (μg/gm) | Number of animals |
|-------------------------|-------------|------------------|
|                         |             |                  |
| 3                       | 1           | 3                |
| 5                       | 0.5         | 4                |
| 6                       | 0.35        | 2                |
| 6                       | 0.25        | 1                |
| 12                      | 0.1         | 0                |

Table 2. Results of the hot plate test for HDP-2 (0.1 μg/gm) in CD-1 mice.

| Animal group | Time (seconds) spent on the hot plate before: |
|--------------|---------------------------------------------|
|              | First forepaw licking | First hindpaw licking | First jump |
| HDP-2 (n = 12) | 6.7 ± 0.7 | 21.3 ± 0.7* | 41.6 ± 1.8* |
| Control (n = 15) | 7.7 ± 0.4 | 14.6 ± 1 | 31.5 ± 1.7 |

*Significant difference (P < 0.05 by Student’s t-test) relative to control.
The toxicity of a heterodimeric PLA2 from V. nikolskii venom for mice was determined. Its LD₅₀ (0.38μg/gm) is close to those obtained for other heterodimeric PLA2s.

- In the hot plate test HDP-2 increased the time before the first hind paw licking and the first jump at the maximal tolerated dose of 0.1μg/gm. However, this effect may be explained by a decrease in locomotor activity rather than analgesic activity of HDP-2.

- Comparison of the analgesic effect produced by crotoxin with that of HDP-2 indicates an important role of acidic subunits in the analgesic activity of heterodimeric PLA2.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATION

PLA2; phospholipase A2

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