Current Topics in Peptide & Protein Research
Vol. 23, 2022

Original Article

Influence of *Vipera berus berus* and *Vipera berus nikolskii* venom on protein-peptide profile in the liver, kidneys and small intestine of rats

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ABSTRACT

The present study aims to investigate the effects of *Vipera berus berus* and *Vipera berus nikolskii* venom on protein homeostasis in rats' liver, kidneys, and small intestine. The animals (a total of 65 albino non-linear male rats) were injected intraperitoneal with crude venom of *V. berus berus* (1.57 $\mu g \cdot g^{-1}$ of body weight) and *V. berus nikolskii* (0.97 $\mu g \cdot g^{-1}$ of body weight). The presence of active enzymes was evaluated by zymography. The level of low-molecular-weight substances was detected at 210, 254, 280 nm. The protein profile was analyzed by polyacrylamide gel electrophoresis; the molecular weight of peptides was estimated by size exclusion chromatography. It was found that snake venom caused disturbances in the protein homeostasis in all organs, manifested by a decrease in the total protein level and changes in the protein composition. An increase in the level of proteins with a molecular weight of less than 30 kDa was found, simultaneously with a decrease in the level of proteins with a molecular weight of more than 100 kDa. The accumulation of low-molecular-weight substances of various nature was also revealed. The administration of snake venom caused an increase in the activity of constitutive enzymes and the appearance of active enzymes that were not found in these organs under physiological conditions.

KEYWORDS: snake venom, protein-peptide composition, total proteolytic activity, liver, kidneys, small intestine.

INTRODUCTION

The snakes *V. berus berus* and *V. berus nikolskii* are widely distributed in Europe. These snakes are responsible for most snakebite accidents. The local effects of *V. berus berus* bites include local pain, severe swelling, blistering, necrosis, and variable non-specific effects [1, 2]. Bites of these snakes are seldom fatal; however they may cause serious disorders in the functional activity of different organs. This affects victims' quality of life and can cause future health problems. Considering the results of clinical studies, the liver and kidneys are among the target organs of snake venom action. Severe hepatocellular injuries, hepatocyte necrosis or/and apoptosis,

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mesangiolysis, glomerulonephritis, vasculitis, tubular necrosis, and interstitial nephritis are usually observed in response to snake venom [3, 4]. Despite advances in understanding the mechanisms of hepatotoxicity and nephrotoxicity, much remains to be learned on the triggers involved in the initiation and progression of these pathological events in response to snake venom. We suggest that the toxic effect of *V. berus berus* and *V. berus nikolskii* can be partly realized through influences on protein homeostasis. Protein homeostasis is recognized to play an important role in maintaining overall metabolism [5]. Any disorders of protein homeostasis can potentially lead to pathological consequences. Venom of *V. berus berus* and *V. berus nikolskii* contains proteins and peptides with diverse functional effects that may have an influence on protein homeostasis. The toxic effect of venoms can be directly caused by proteolytic enzymes, especially metalloproteases, present in the venom. The additional mechanism involves the influence of the components of the snake venom on factors that, in turn, can directly or indirectly affect the activity/content of the cell proteases. Thus, it is interesting to study the effect of crude snake venom on the protein-peptide profile and overall proteolytic state in the liver, kidneys, and small intestine of rats, unveiling the molecular mechanisms through which venom of *V. berus berus* and *V. berus nikolskii* realizes toxic effect.

MATERIALS AND METHODS

Venom

Lyophilized *V. berus berus* and *V. berus nikolskii* crude venoms were obtained from V. N. Karazin Kharkiv National University (Kharkiv, Ukraine), kept at −20 °C, dissolved in saline immediately before experiments, centrifuged at 10,000 g for 15 minutes, and the supernatant was used.

Animals

A total of 65 albino non-linear male rats was used in the study. All experiments on animals were performed in compliance with international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The Ethical Committee approved the study of Taras Shevchenko National University of Kyiv. The experiments were started after 7 days of animal acclimation in the animal facility of Taras Shevchenko National University of Kyiv, maintained under constant conditions of temperature (22 ± 3 °C), humidity (60 ± 5%), and light (12 h light/12 h dark cycle). Standard rodent food and water were provided *ad libitum*. Rats were randomly divided into three groups of ten animals in each group. The first group served as a control and the rats of this group were injected intraperitoneally (i.p.) with saline solution. The rats of the second and third groups were injected i.p. with a median lethal dose (LD50) according to [6]. The rats of the second group were injected i.p. with LD50 (1.576 $\mu g \cdot g^{-1}$ of body weight) of *V. berus berus* venom in saline solution. The rats of the third group were injected i.p. with LD50 (0.972 $\mu g \cdot g^{-1}$ of body weight) of *V. berus nikolskii* venom in saline solution. After 24 hours, the surviving animals were killed by cervical dislocation. The liver, kidneys, and small intestine were immediately collected.

Protein determination

The protein concentration was determined by the Bradford method [7] (1976) using crystalline bovine serum albumin as a standard.

Low-molecular-weight substances estimation

The fraction of LMWS was obtained according to the method described by [8]. The liver/kidneys/small intestine homogenates were mixed with 1.2 M $HClO_4$ at a 1:1 (v/v) ratio to precipitate the proteins. After centrifugation at 10000 g for 20 min at +4 °C, the supernatants were neutralized by 5 M KOH to pH 7.0 and the samples were again subjected to centrifugation. After adding ethanol to the final concentration of 80%, the samples were kept at +4 °C for 30 min and centrifuged (10,000 g for 5 min). The optical density of the supernatants was determined with a spectrophotometer Smart SpecTMPlus (BioRad, USA) at 210 nm, 238 nm, and 254 nm. The level of LMWS was expressed as rel. units per g of tissue.

Analysis of peptide fractions by size-exclusion chromatography

The peptide fractions were analyzed by size exclusion chromatography on Sephadex G

15 column (BioRad, USA). The column was pre-equilibrated with 0.05 M Tris-HCl (pH 7.4) containing 0.13 M NaCl. The samples were loaded at a flow rate of 30 mL per hour. The areas under the peaks of chromatographic curves were calculated using the OriginLab program (v.9.1). The molecular weight of peptides was estimated using a calibration curve. For this purpose, the column was previously calibrated with a standard marker solution containing lysozyme (14.3 kD), insulin (5.7 kDa), and vitamin B12 (1.35 kDa).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Zymographic assay

SDS-PAGE was carried out according to [9]. Samples were prepared by mixing with sample buffer (0.005 M Tris-HCl (pH 8.8), 2% SDS, 5% sucrose, and 0.02% bromophenol blue) at the ratio of 1:1 (v/v). Samples were heated at +95 ºC for 1 min before loading into the gel. The gels were stained with 2.5% Coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, and 15% (v/v) isopropanol for 30 min. Apparent molecular weights of proteins were estimated using a protein calibration mixture (BioRad, USA). Zymography was carried out according to [10]. The separating gel solution (12%) was polymerized in the presence of gelatin (1 mg per mL of gel solution). Samples were not subjected to heating before loading in the gel. After electrophoresis was done, the gels were soaked in 2.5% Triton X-100 solution with shaking (30 min at +25 °C) for SDS removal and renaturation of proteins. The gels were washed with distilled water for 10 min to remove Triton X-100 and then were incubated in 50 mM Tris-HCl (pH 7.5) at +37 °C for 12 h. The digested bands were visualized as the nonstained regions of the gel. TotalLab 2.04 program was used to analyze the electropherograms. The represented electropherogram and zymogram are typical for the series of the repeated experiments (at least three in each series).

Statistical analysis

The data of biochemical estimations were reported as mean ± SEM for each group (n = 10). Statistical analyses were performed using a one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Total protein level

According to the obtained data (Table 1), the single IP injection of *V. berus berus* and *V. berus nikolskii* venoms caused a decrease in the level of proteins in the liver, kidneys, and small intestine. The total protein level in the liver, kidneys and small intestine of the rats administrated with *V. berus berus* venom were 1.43 times, 1.59 times, and 1.11 times lower than those in the corresponding controls. The injection of *V. berus nikolskii* venom resulted in more pronounced changes in the protein level – this parameter decreased 2.23 times in the liver, 1.06 times in the kidneys, and 1.41 times in the small intestine compared with the control animals.

Protein composition

As shown in Table 2, the protein composition in the liver, kidneys, and small intestine of the control animals was represented by protein fractions with the molecular weight of ≤ 10 kDa, 10-35 kDa, 35-67 kDa, 67-100 kDa, and 100-150 kDa. The injection of snake venoms caused the changes in the level of proteins of different molecular weights. The significant decrease in the level of high-molecular-weight proteins (100-150 kDa) was revealed in all organs in response to the injection of *V. berus nikolskii* venom. This was accompanied by the accumulation of 10-35 kDa proteins in the liver, small intestine, and proteins with a molecular weight of less than 10 kDa in the kidneys. The venom injection of *V. berus berus* caused an increase in the level of 10-35 kDa proteins - 2.11 times in the liver, 1.53 times in the kidneys, and 2 times in the small intestine. The fractions of proteins with a molecular weight of 67-100 kDa were not found in the liver and kidneys. In contrast to the effects observed after the injection of *V. berus nikolskii* venom, the injection of *V. berus berus* venom caused an increase in the level of proteins with a molecular weight of 100-150 kDa. This parameter was twice as high in the liver and 1.43 times higher in the kidneys.

Table 1. The total protein levels in the liver, kidneys and small intestine of the rats injected with snake venom.

Organs	Groups	Level of proteins, mg per g of tissue
Liver	Control	112.23 ± 1.87
	V. berus berus	78.15 ± 3.53*
	V. berus nikolskii	50.19 ± 2.53*
Kidneys	Control	90.01 ± 3.16
	V. berus berus	86.93 ± 5.99
	V. berus nikolskii	56.38 ± 3.39*
Small intestine	Control	66.19 ± 1.74
	V. berus berus	59.20 ± 2.20*
	V. berus nikolskii	47.17 ± 2.70*

Values are expressed as mean ± SEM (n = 10); *p<0.05 significantly different from the corresponding control.

Table 2. Protein composition in the liver, kidneys and small intestine of the rats injected with snake venom.

Organs	Groups	Protein fractions, kDa					
		>150	150-100	100-67	67-35	35-10	<10
		Band, % (number of fractions)					
Liver	Control	-	4.27 (1)	18.86 (3)	28.02 (3)	29.25 (2)	19.61 (1)
	V. berus berus	-	8.96 (1)	-	28.99 (2)	62.06 (2)	-
	V. berus nikolskii	-	-	4.32 (1)	20.63 (3)	57.96 (3)	17.09 (1)
Kidneys	Control	-	16.65 (1)	9.46 (2)	22.12 (2)	34.15 (2)	17.62 (2)
	V. berus berus	-	23.03 (1)	-	-	52.30 (4)	24.66 (2)
	V. berus nikolskii	-	-	12.10 (3)	33.81 (3)	17.69 (2)	36.42 (2)
Small intestine	Control	-	20.12 (1)	-	8.85 (2)	29.39 (2)	41.64 (2)
	V. berus berus	-	12.49 (1)	-	28.32 (4)	59.18 (4)	12.49 (1)
	V. berus nikolskii	-	0.95 (1)	7.61 (6)	10.00 (6)	37.46 (5)	43.58 (2)

Values are expressed as mean ± SEM (n = 10).

Level of LMWS

The level of LMWS in the liver, kidneys and small intestine of the rats injected with snake venoms was determined. To provide a more complete assessment of the nature of LMWS, measurements were carried out at wavelengths of 210 nm (reflects the presence of peptides), 238 nm (reflects the presence mainly of non-aromatic peptides), and 254 nm (reflects the presence of non-aromatic sulfur-containing molecules, as well as purine bases and some nucleotides). As seen from Table 3, the liver, kidneys, and small intestine of the control animals have the fractions of LMWS that were registered at all three wavelengths. The level of LMWS registered at 210 nm was found to be significantly higher than the levels of LMWS registered at 238 nm and 254 nm. The injection of snake venoms caused an increase in the level of LMWS. The most pronounced changes were found in the rats treated with *V. berus nikolskii* venom. In the liver, the level of LMWS registered at 210 nm, 238 nm, and 254 nm increased 1.65 times, 16.71 times, and 2.64 times compared to the corresponding controls. These parameters in the kidneys were 1.24 times (for wavelength 210 nm), 3.64 times (for wavelength 238 nm), and 3.13 times (for wavelength 254 nm) higher than those in the control animals. In the small intestine, the level of LMWS registered at 210 nm, 238 nm, and 254 nm was 1.41 times, 4.18, and 5 times higher than the results in the control rats.

Identification of active proteases

The presence of active enzymes in the tissue of the liver, kidneys, and small intestine was investigated by the method of zymography. The obtained zymograms were analyzed using TotalLab 2.04 program and the results are presented in Table 4. The injection of snake venom caused the appearance of active enzymes that were not found in the control samples. The appearance of active enzymes with molecular weight in the range of 100-150 kDa were found in the liver and kidneys of the animals administrated with *V. berus berus* and *V. berus nikolskii* venom. The clear zones at the region of 10-35 kDa were also detected in the kidneys in response to the injection of *V. berus nikolskii* venom.

Analysis of peptide component of the fraction of LMWS

The fraction of LMWS was subjected to size exclusion chromatography to analyze the peptide composition. According to the obtained data (Table 5), the peptide fractions in the kidneys and

Table 3. Level of LMWS in the liver, kidneys and small intestine of the rats injected with snake venom.

Organs	Groups	LMWS, rel. units per g of tissue		
		Registered at 210 nm	Registered at 238 nm	Registered at 254 nm
Liver	Control	11.05 ± 0.97	1.30 ± 0.08	0.53 ± 0.03
	V. berus berus	15.63 ± 1.87*	13.17 ± 2.69*	10.80 ± 1.55*
	V. berus nikolskii	18.7 ± 1.22*	21.76 ± 0.88*	14.02 ± 0.26*
Kidneys	Control	18.89 ± 1.92	6.31 ± 0.05	4.82 ± 0.85
	V. berus berus	19.65 ± 1.34	8.71 ± 0.60*	7.56 ± 1.16*
	V. berus nikolskii	23.36 ± 3.01*	23.14 ± 2.87*	15.13 ± 0.88*
Small intestine	Control	19.33 ± 0.13	5.95 ± 0.19	3.54 ± 0.76
	V. berus berus	23.10 ± 2.30*	11.80 ± 02.50*	9.30 ± 1.80*
	V. berus nikolskii	27.30 ± 3.80*	24.90 ± 3.90*	17.90 ± 2.20*

Values are expressed as mean ± SEM (n = 10); *p<0.05 significantly different from the corresponding control.

Table 4. Distribution of active enzymes in the liver, kidneys, and small intestine of the rats injected with snake venom.

Organs	Groups	Protein fractions, kDa					
		>150	150-100	100-67	67-35	35-10	<10
		Band, % (number of fractions)					
Liver	Control	-	-	57.00 (1)	43.02 (1)	-	-
	V. berus berus	-	37.4 (1)	29.22 (1)	33.38 (2)	-	-
	V. berus nikolskii	-	63.16 (1)	7.58 (1)	29.26 (3)	-	-
Kidneys	Control	-	16.65 (1)	9.46 (2)	22.12 (2)	34.15 (2)	17.62 (2)
	V. berus berus	-	8.81 (1)	88.51 (4)	2.67 (1)	-	-
	V. berus nikolskii	-	8.71 (1)	81.74 (3)	9.52 (1)	0.03 (1)	-
Small intestine	Control	-	-	27.38 (3)	40.63 (2)	31.99 (1)	-
	V. berus berus	-	-	76.50 (3)	8.47 (4)	15.02 (1)	-
	V. berus nikolskii	-	-	46.58 (3)	30.42 (3)	22.71 (1)	-

Values are expressed as mean ± SEM (n = 10).

small intestine of the control animals were represented by four main fractions. The liver was found to have three main fractions of peptides. As seen from Table 5, the liver of the control animals contained peptides with molecular weight from 1107 Da to 1986 Da; the kidneys of the control animals contained peptides with molecular weight from 1121 Da to 2262 Da, and the small intestine contained peptides with molecular weight from 820 Da to 2355 Da. The injection of snake venoms led to the appearance of intermediate-molecular weight peptides. The most pronounced changes in the composition of the peptide fractions were found in the rats treated with *V. berus nikolskii* venom. In this case, the peptide fractions increased to five fractions in the liver and kidneys and seven fractions in the small intestine.

DISCUSSION

Snake venoms are complex and variable mixtures of bioactive components that realize their effects through various mechanisms. Since snake venoms include proteases, proteins in prey tissues may be among the potential targets for snake venom enzymes [11, 12]. The stability of tissue protein composition is known to be an important factor in maintaining overall homeostasis, and any disruption of protein metabolism can lead to severe consequences and sometimes pathological states. In the current study, we established that injection of *V. berus berus* and *V. berus nikolskii* venom affected protein homeostasis in the liver, kidneys, and small intestine. This manifested as a decrease in the total protein levels in all studied organs. Our results are consistent with those of

Table 5. Peptide composition of the liver, kidneys, and small intestine of the rats injected with snake venom.

Organs	Groups	Molecular weight, Da	Area under peak, r.u
Liver	Control	1986	0.102
		1378	0.073
		1107	0.005
	V. berus berus	2019	0.064
		1391	0.100
	V. berus nikolskii	1910	0.156
		1307	0.078
		1067	0.004
		1026	0.003
		798	0.007
Kidneys	Control	2262	0.216
		1489	0.148
		1209	0.007
		1121	0.042
	V. berus berus	2368	0.112
		1972	0.166
		1416	0.142
		1094	0.045
	V. berus nikolskii	2324	0.187
		1521	0.116
		1230	0.012
		924	0.009
		837	0.001
Small intestine	Control	2355	0.029
		1992	0.146
		1407	0.102
		820	0.014
	V. berus berus	1927	0.237
		1296	0.133
		1107	0.005
		1105	0.142
		769	0.016
	V. berus nikolskii	2199	0.018
		1890	0.284
		1330	0.148
		784	0.011
		959	0.003
		956	0.002
		1073	0.004

Values are expressed as mean ± SEM (n = 10).

other researchers. According to [13], protein breakdown in the liver of rats injected with *Naja naja* snake venom prevails over their synthesis. The decrease in the protein level in the organs may be the result of cell destruction due to necrosis and the release of the cellular content into extracellular space [14]. Additionally, snake venom-induced vascular permeability may contribute to the loss of proteins in the tissues [15].

The protein profile in the animals injected with snake venom was also investigated in the liver, kidneys, and small intestine. Our findings revealed some changes in the protein composition that were more pronounced in the rats administrated with *V. berus nikolskii* venom. Summing up, an increase in the content of proteins with a molecular weight of less than 30 kDa was detected, simultaneously with a decrease in the level of proteins with a molecular weight of more than 100 kDa. Such distribution of proteins is characteristic of the enzyme-mediated degradation of proteins and can be explained by the activation of proteases of the victim in response to the administration of snake venom, and/or it can be the result of the direct action of snake venom enzymes on tissue proteins. Snake proteinases, namely metalloproteinase can attack basement membrane proteins or extracellular matrix components in the tissue of victims, increasing the spread of venom in tissues. Enzymatic degradation of proteins may lead to the appearance of molecules that have lost their biological activity and therefore cannot perform their inherent functions. At the same time, some of these truncated molecules can retain enzymatic activity due to the preservation of the active site [16]. However, being structurally defective, they can avoid inhibition by canonical inhibitors. In light of the obtained results, the intensity of proteolysis in the rats injected with snake venom was investigated in the liver, kidneys, and small intestine. Our data revealed that the administration of snake venom caused both an increase in the activity of enzymes and the appearance of active enzymes that are not found in these organs under physiological conditions. Changes in the activity of tissue proteases are considered as one of the mechanisms of harmful action of venomous snakes. The exact molecular mechanisms by which snake venom triggers the protease activation in the tissue of victims are still unclear. It can be assumed that the changes in the proteolytic pattern in the liver, kidneys, and small intestine are partly associated with inflammation and oxidative stress that develop in response to exposure to snake venom. Reactive oxygen species, through the regulation of redox-sensitive pathways, affect the expression of both proteases and their inhibitors, possibly leading to abnormal protease activation [17]. In addition, the enzymatic components of snake venom can directly activate zymogens in the victim’s tissue into active forms. Uncontrolled and excessive proteolysis can provoke cell dysfunction and even cell death, mainly due to necrosis.

According to the literature, snake venom envenoming is characterized by the development of acute intoxication. Among the manifestations of intoxication are accumulation of low molecular substances (LMWS) in tissues and biological fluids of victims, which serves as a marker of this condition [18, 19]. The fraction of LMWS is heterogeneous and consists of substances with a molecular weight up to 5000 Da [20]. Most of these molecules are typical of normal metabolism and are detected at minimal concentrations under physiological conditions. However, the increase in their concentration above the physiological values and/or the appearance of the excess number of products of impaired metabolism can be potentially harmful to cells, as they affect the biochemical processes [21]. To clarify whether the venom of *V. berus berus* and *V. berus nikolskii* cause the appearance of LMWS, the levels of these substances in the liver, kidneys, and small intestine were estimated. The accumulation of LMWS, especially peptides, clearly indicates the intensification of the catabolic process and can be evidence of the intoxication state in the organs of rats administrated with snake venom. Since the most significant changes were detected in the peptide component of LMWS, the peptide fraction was further analyzed by size exclusion chromatography. The appearance of peptides of intermediate molecular weight in the rats’ liver, kidneys, and small intestine is consistent with the decrease in the protein level. It may indirectly indicate the increase in the proteolytic degradation of proteins. It should be noted that changes in the qualitative and quantitative composition of peptides are not only the result of metabolic disorders mediated by snake venoms but it can be among the reasons underlying further complications in victims. Taking into account modern concepts, the sum of peptides in the tissue - the peptide pool - is actively involved in the maintenance and regulation of tissue homeostasis. Under physiological conditions, peptides regulate growth, remodeling, repair, and development of tissues [22]. However, intensification of proteolysis simultaneously with impairment of peptide clearance can lead to the accumulation of peptides that can have biological activity and affect cellular processes. Being structurally similar to naturally occurring peptides, these bioactive peptides can bind to cell receptors influencing the intracellular metabolism. Peptides can increase venom toxicity by triggering and amplifying inflammation or inducing the transcription of enzymes, namely, metalloproteases.

CONCLUSION

In conclusion, for the first time, this research investigated the influence of snake venom on protein-peptides profile in target organs. Changes in the protein composition of the liver, kidneys, and small intestine confirm the harmful effect of the venom of *V. berus berus* and *V. berus nikolskii*. On the other hand, these disorders can be part of the mechanisms underpinning the development of pathological consequences in response to the injection of snake venom.

ACKNOWLEDGEMENTS

The authors would like to express their deepest gratitude to Zinenko Oleksandr for providing the venoms of *V. berus berus* and *V. berus nikolskii*.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflict of interests regarding the publication of this manuscript.

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