In Vivo Effects of Bothrops jararaca Venom on Metabolic Profile and on Muscle Protein Metabolism in Rats

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Abstract. This study investigated the in vivo effects of the Bothrops jararaca venom (BjV) on general metabolic profile and specifically, on muscle protein metabolism in rats. The crude venom (0.4 mg/kg body weight, IV) was infused in awake rats, and plasma activity of enzymes and metabolites levels were determined after 1, 2, 3, and 4 hours. BjV increased urea, lactate, and activities of creatine kinase, lactate dehydrogenase, and aspartate aminotransferase after 4 hours. The content of liver glycogen was reduced by BjV. Protein metabolism was evaluated by means of microdialysis technique and in isolated muscles. BjV induced increase in the muscle interstitial-arterial tyrosine concentration difference, indicating a high protein catabolism. The myotoxicity induced by this venom is associated with reduction of protein synthesis and increase in rates of overall proteolysis, which was accompanied by activation of lysosomal and ubiquitin-proteasome systems without changes in protein levels of cathepsins and ubiquitin-protein conjugates.

INTRODUCTION

Bothrops jararaca is one of the most important species responsible for venomous snakebites in South America. Envenomation by this species is characterized by prominent local tissue damage and systemic disturbances such as hemorrhage, coagulopathies, hypotension, and renal alterations. Acute renal failure (ARF) is the most important toxic effect observed in humans after Bothrops poisoning, and it is responsible for a large number of deaths in patients surviving the initial consequences of the bite.

The pathogenesis of systemic alterations induced by Bothrops envenomation involves both the direct action of venom components on the tissues and the release of endogenous mediators such as histamine, bradykinin, eicosanoids, NO, and cytokines. Although the biological, pharmacologic, and immunologic properties of the venom of genus Bothrops are well established, very little is known about the biochemical and metabolic effects induced by Bothrops poisoning in vivo. This is at least in part because of the lack of adequate in vivo experimental models for the study of this complex clinical picture. Using a reproducible nephrotoxicity model of uremia, Burdmann and others have shown that intravenous infusion of sublethal doses of B. jararaca venom (BjV) into rats resulted in a functional and morphologic lesion that resembled snakebite-induced ARF in humans. It is well established that acute and chronic renal failure cause metabolic disturbances in different organs and tissues, especially in skeletal muscle. It has been shown, in acute experimental models of uremia, alterations in glucose and protein metabolism that could contribute to the loss of muscle proteins, but the exact mechanism by which snake venoms affect the rate of protein metabolism in skeletal muscles from humans and animals has never been studied.

Skeletal muscle protein mass depends on the balance between synthesis and degradation. In all tissues, the majority of intracellular proteins are degraded by the ubiquitin-proteasome system (UPS). However, extracellular proteins and some cell surface proteins are taken up by endocytosis and can also be degraded within lysosomes, which contain several acid-optimal proteases, including cathepsins B, D, H, and L. Another important proteolytic system is the Ca²⁺-dependent process that is activated by calcium and involves the cysteine proteases termed calpains. The mechanisms by which hormones, nutrients, cytokines, toxins, and venoms activate proteolysis and the precise contribution of each proteolytic system to the enhanced breakdown rates observed in skeletal muscles in physiologic and pathologic conditions are still unclear.

The purpose of this study was therefore 2-fold. 1) To investigate the general metabolic profile in an experimental model of snakebite-induced ARF. For this purpose, the crude venom of B. jararaca (0.4 mg/kg body weight, IV) or saline was slowly infused in awake Wistar rats, and plasma activity of enzymes (creatine kinase [CK], lactate dehydrogenase [LDH], aspartate aminotransferase [AST], and alanine aminotransferase [ALT]) and metabolites levels (glucose, lactate, free fatty acids, urea, and creatinine) were determined after 1, 2, 3, and 4 hours. 2) To examine the effect of the venom on skeletal muscle protein metabolism in vivo, the interstitial tyrosine concentration, rate of protein synthesis, activity of proteolytic processes (lysosomal, Ca²⁺-dependent, and ubiquitin-proteasome system [UPS]), and the content of cathepsins (B and L) and ubiquitin-protein conjugates were measured. The mean arterial pressure, muscle blood flow, and the content of lipids and glycogen in liver and skeletal muscle from envenomated rats by B. jararaca are also reported.

MATERIALS AND METHODS

Animals. Adult male Wistar rats, weighing 250–300 g, were used in all experiments. For in vitro experiments, young rats, weighing ~70 g, were used because the incubation procedure required intact muscles sufficiently thin to allow an adequate diffusion of metabolites and oxygen. Rats were housed in a room with a 12–12 hour light-dark cycle and were given free access to water and normal laboratory chow diet for at least 1 day before the beginning of the experiments, which were per-

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formed at 8:00 AM. The protocol studies were approved by the Ethical Commission of Ethics in Animal Research (CETEA) from School of Medicine of São José do Rio Preto and agree with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA).

**Venom.** Lyophilized *B. jararaca* crude venom was obtained from Butantan Institute (São Paulo, Brazil), kept at −20°C, and dissolved in saline immediately before experiments.

The rats received a 10-minute intravenous infusion of 0.4 mg of *B. jararaca* venom (BjV) per kilogram body weight or the same volume of saline at a rate of 0.1 mL/min. This dose was based on a previous study, in which BjV was infused in rats, resulting in a venom blood concentration of ~10 μg/mL (the average amount of venom inoculated by *B. jararaca* into humans).

**Metabolic parameters.** One day before the experiment, the left common carotid artery and right jugular vein were catheterized with a polyethylene tube (PE-50; Becton Dickinson, Sparks, MD) under xylazine and ketamine anesthesia (10 and 85 mg/kg body weight, respectively, IP). The free ends of catheters were tunneled under the skin to the back of the neck, which were exteriorized and sealed with stainless steel plugs. Glucose, lactate, free fatty acid (FFA), urea, creatinine, CK, LDH, AST, and ALT were measured in arterial blood samples and measurement of blood pressure were taken 2 and 4 hours of the experiment. Tyrosine was assayed as previously described. To measure the participation of the different proteolytic pathways, muscles were incubated as described in detail previously.

**Rates of protein degradation.** Muscles were incubated at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, equilibrated with 95% O2-5% CO2, containing glucose (5 mmol/L) and in the presence of cycloheximide (0.5 mmol/L) into the incubation medium to prevent protein synthesis and reincorporation of tyrosine back into proteins. Tissues were preincubated for 1 hour and incubated for 2 hours in fresh medium of identical composition.

The rates of overall proteolysis and of the different proteolytic systems were measured by following the rate of tyrosine release into the medium. Because muscle cannot synthesize or degrade tyrosine, its release reflects the rate of protein breakdown. Tyrosine was assayed as previously described. To measure the participation of the different proteolytic pathways, muscles were incubated as described in detail previously.

**Rates of protein synthesis.** Muscles were rapidly dissected, maintained at approximately resting length, and incubated at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, equilibrated with 95% O2-5% CO2, containing glucose (5 mmol/L) and all 20 amino acids at concentrations similar to those of rat plasma. The procedure used for measurement of rates of protein synthesis in muscles from saline and envenomated rats was as previously described.

**Western blotting analysis.** Cathepsin B and L (total proteins) and ubiquitin (Ub)-protein conjugates (myofibrillar fraction) were measured by Western blotting analysis. For cathepsins, EDL muscles were harvested and homogenized in 20 mmol/L Tris-HCl and 5 mmol/L EDTA, pH 7.5, in the presence of 1 mg/mL leupeptin, 100 mg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/mL aprotinin. The homogenate was centrifuged at 14,000g at 4°C for 20 minutes, retaining the supernatant that was stored at −80°C. For Ub-protein conjugates, EDL muscles were harvested and homogenized in 5 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 5 mmol/L dithiothreitol (DTT), 50 μmol/L MG132 in the presence of 1 mg/mL leupeptin, 100 mg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/mL aprotinin. Myofibrillar proteins were pelleted by centrifugation at 1,500g for 5 minutes at 4°C. Pellets were washed three times in the same buffer containing 1% Triton X-100 and resuspended in 8 mol/L urea and 50 mmol/L Tris-HCl, pH 7.5. Supernatants were stored at −80°C. Total protein content and myofibrillar protein content were determined in duplicate by the method of Lowry and others using bovine serum albumin (BSA) as a standard. An equal volume of samples buffer (20% glycerol, 125 mmol/L Tris-HCl, 4% SDS, 100 mmol/L dithiothreitol, and 0.02% bromophenol blue, pH 6.8) was added to homogenate, and the mixture was boiled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–12% acrylamide gels. Gels were electroblotted on nitrocellulose membranes, and the primary antibodies were used to detect the corresponding protein levels: goat anti-cathepsin B and L (1:100) and rabbit anti-Ub-protein conjugates (1:1,000). Primary antibody was detected by peroxidase-conjugated secondary antibody (1:10,000) and visualized by enhanced chemiluminescence reagents. Band intensities were quantified using the LabImage Program.
2.7.1). The Ub-protein conjugates were assessed as the sum of densitometric values of bands with molecular weight higher than 55 kd. The densitometry of bands was analyzed three times, and the average value was reported.

Hemodynamic parameters. Mean arterial pressure. The left carotid artery from awake or anesthetized rats was catheterized and connected to a pressure transducer (Braille Biomedical, São José do Rio Preto, Brazil) for measurement of mean arterial pressure (MAP). The MAP was recorded every 15 minutes throughout the experiment.

Muscle blood flow. Blood flow changes around the microdialysis probe were measured using the ethanol method described by Hickner and others.23 Each experiment included 120 minutes of perfusion with isotonic saline containing 0.5% BSA, 1 mmol/L glucose, and 5 mmol/L ethanol. Dialysate samples were collected every 15 minutes throughout the experiment.

Calculations. The in vivo recovery of the microdialysis catheters was determined in all samples and was calculated from the ratio of tyrosine concentration in the dialysate/perfusate. To calibrate the catheters, [14C]-tyrosine (~2,500 DPM) was added to the perfusate, and the fractional extraction of radioactivity (% recovered at each period) was measured. The following formula was used to calculate the interstitial tyrosine concentration:

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[Tyr]_{\text{interstitial}} = \frac{[Tyr]_{\text{dialysate}} - [Tyr]_{\text{perfusate}}}{\text{recovery}} + [Tyr]_{\text{dialysate}}
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Changes in blood flow are expressed as ethanol outflow/inflow ratio, that is, ethanol concentration in the dialysate/perfusate.

Biochemical determinations. Plasma glucose, lactate, and ethanol concentrations were determined using the YSI 2700 select biochemical analyzer (Yellow Springs, OH). Commercial kits were used to measure urea, creatinine, and all enzymes spectrophotometrically (Drake Electronic and Trade, São José do Rio Preto, Brazil). Determination of plasma FFA followed the method of Dole and Meinertz.24 Liver and muscle glycogen was measured by the method of Carroll and others.25 The content of lipids in liver was determined gravimetrically after extraction by the procedure of Folch and others.26

Statistical analysis. Data are presented as means ± SE. The Student t test or Mann-Whitney U test was chosen for analysis of numerical variables with normal or non-normal distributions, respectively. P < 0.05 was taken as the criterion of significance.

RESULTS

Two hours after envenomation, rats presented dark reddish-brown urine, suggesting the presence of hematuria and hemoglobinuria, events that have been shown to occur in rats infused with the same dose of the venom used in this study.5 In the 24 hours after venom infusion, no deaths were observed.

Systemic effects of BjV on metabolic parameters in awake and anesthetized rats. Table 1 shows the mean arterial pressure (MAP) and plasma levels of glucose, lactate, FFA, urea, and creatinine 1, 2, 3, and 4 hours after saline or BjV infusion in rats. The envenomation induced a small (15%) but statistically significant increase in the MAP after 1 and 2 hours. Plasma levels of lactate increased (46%) after 1 hour, did not change after 2 and 3 hours, and reached the highest value (144%) 4 hours after venom infusion. BjV caused a time-dependent increase of uremia and increased plasma creatinine levels by 39% after 4 hours from infusion. Plasma levels of glucose and FFA were not altered by Bothrops poisoning.

In an attempt to determine the extension of tissue damage induced by BjV, several plasma enzymes activities were measured. Envenomation caused a marked and progressive increase in plasma levels of CK, LDH, and transaminases (Figure 1). The CK activity in envenomated rats increased 100% and 7-fold after 1 and 4 hours, respectively. The LDH activity drastically increased (~10-fold) after 1 hour and reached the highest value (16-fold) 4 hours after venom administration. Figure 1B shows that the venom infusion induced a 74% and 78% increase in ALT activity after 3 and 4 hours, respectively. A significant increase of 66% and 2-fold in AST activity was observed 1 and 4 hours after Bothrops envenomation, respectively.

BjV infusion in anesthetized rats decreased the content of liver glycogen after 2 hours (2.72 ± 0.30% versus 4.03 ± 0.15% in controls; P < 0.05) but did not significantly affect muscle (0.49 ± 0.03% versus 0.48 ± 0.02% in controls) glycogen levels or the content of total lipids in liver (5.16 ± 0.12% versus 4.81 ± 0.18% in controls).

Effects of BjV on muscle interstitial tyrosine levels and muscle blood flow. Microdialysis technique was used to estimate skeletal muscle protein metabolism in vivo. The mean
fractional outflux of [14C]-tyrosine (relative recovery) through the microdialysis catheter in skeletal muscle from saline and experimental group did not change throughout the experiment and was not affected by venom administration (data not shown). This suggests that these data were obtained in steady-state conditions.

The mean tyrosine concentration in the muscle interstitial fluid from saline-infused rats (Figure 2A) was similar to that found in previous experiments from our laboratory in control non-treated rats and in other studies with humans. This parameter increased 37% 2 hours and 59% 4 hours after envenomation (Figure 2A). As shown in Figure 2, venom infusion induced an increase in muscle interstitial tyrosine concentration (37–59%), in arterial plasma tyrosine levels (61%) only after 4 hours, and in interstitial-arterial (I-A) tyrosine concentration difference (3-fold). In these experiments, BjV-induced myotoxicity was confirmed by measurement of plasma CK levels that were 2.5-fold higher than in saline-treated rats.

The muscle blood flow (MBF) must always be monitored in microdialysis experiments for a suitable interpretation of the data, because hemodynamic changes affect the clearance of metabolites in situ and consequently modify the interstitial concentration. MBF (Figure 3) and MAP (data not shown) did not change along experimental period either in venom or in saline group.

**Effects of BjV on the activity of proteolytic pathways, content of proteases, and the rate of protein synthesis.** Rates of overall proteolysis in EDL muscles increased (32%) 4 hours after Bothrops poisoning (Figure 4). The rise in rates of pro-
tein degradation was accompanied by an increased activity of lysosomal (113%) and Ub-proteasome–dependent (34%) proteolytic systems, with no significant changes in the activity of Ca²⁺-dependent proteolysis (Figure 4) or ATP-independent proteolysis (data not shown). BjV did not affect either the protein expression level of cathepsin B and L (Figure 5) or Ub-protein conjugates (Figure 6) in EDL muscles.

Rates of protein synthesis in EDL muscles from rats 4 hours after envenomation (six rats, 0.250 ± 0.013 nmol Tyr incorporated/mg⁻¹ · 2 h⁻¹) were 17% lower than in control rats (nine rats, 0.301 ± 0.014 nmol Tyr incorporated/mg⁻¹ · 2 h⁻¹).

DISCUSSION
To study the metabolic effects induced by Bothrops poisoning in awake rats, we used a well-established experimental model that produces functional and morphologic changes similar to those observed in human snakebite-induced ARF. The findings that urea and creatinine acutely increased after the BjV infusion confirmed the acute renal damage in our experimental model and are in agreement with previous studies in which a significant decrease in the glomerular filtration rate and diuresis were observed in anesthetized rats infused with the same dose of the venom used here.

Previous studies have shown that BjV-infused rats decreased or did not change blood pressure. In contrast, this work shows a transient and small increase in the arterial blood pressure induced by intravenous administration of the venom in awake rats, which suggests a stimulatory effect of the sympathetic nervous system. The fact that anesthetized animals have been extensively used in earlier studies helps to explain why this effect has never been reported before. Moreover, this study shows that, when animals were anesthetized, the venom-induced increase in arterial blood pressure observed in awake rats did not appear. It has been reported that the administration of other venoms, mainly scorpion venoms such as Tityus serrulatus, in rats significantly increased the release of catecholamines by the adrenal glands and postganglionic nerve terminals. In agreement with the hypothesis...
that BjV stimulates the release of catecholamines in awake rats, this study showed a decrease in the content of glycogen in liver 2 hours after venom infusion. Despite glycogen mobilization, plasma levels of glucose and muscle glycogen did not change after the envenomation. Considering that plasma lactate levels were increased it could suggest that the rate of glucose utilization by peripheral tissues was increased by venom infusion.

It is well known that Bothrops venom produces marked local effects on skeletal muscle such as edema, hemorrhage, and necrosis\textsuperscript{32–35} that are mediated mainly by metalloproteinases\textsuperscript{4,36} and phospholipases A\textsubscript{2} (PLA\textsubscript{2}).\textsuperscript{37} However, this venom does not seem to induce a systemic myotoxic action similar to the South American Crotaulus.\textsuperscript{38} In earlier studies, Burdmann and others\textsuperscript{5} did not find changes in plasma CK activity in rats infused with BjV and concluded that ischemia and intravascular hemolysis were the most important factors in the etiopathogenesis of ARF. Although we also observed a concomitant increase in plasma LDH, indirect evidence of hemolysis, this study showed a time-dependent increase in CK and AST levels that peaked 4 hours after venom infusion, suggesting that Bothrops venom induces muscle damage. It has also been shown\textsuperscript{39,40} that BjV increases the rates of CK release from isolated skeletal muscles preparations. Based on these findings, it could be suggested that myoglobinuria may be an additional factor in the pathogenesis of the BjV-induced renal lesions. The drastic increase in plasma ALT levels observed 3 and 4 hours after the venom may be caused by lesions from different tissues including liver, kidney, heart, and even skeletal muscle.\textsuperscript{31,42} Similar alterations in plasma transaminases levels have also been described in rats after injection with other snake venoms, including Sistrurus miliarius barbouri\textsuperscript{43} and Bothrops asper.\textsuperscript{44}

Although the myotoxic activity of snake venoms can be monitored indirectly by means of morphologic analysis and plasma CK, LDH, AST, and ALT activities, these approaches often fail to accurately reflect alterations in protein metabolism. In recent studies, we standardized a microdialysis technique to investigate the protein metabolism in rat skeletal muscle.\textsuperscript{27} Direct measurements of tyrosine concentrations in muscle interstitial space by microdialysis provide important additional information on physiologic and pathologic processes in muscle tissues \textit{in vivo}.\textsuperscript{35} In these microdialysis experiments, BjV infusion resulted in a drastic increase in the interstitial-arterial tyrosine concentration difference, indicating net protein degradation in tibialis anterior muscle \textit{in vivo}. Considering that \textit{Bothrops} venom induces hemodynamic disturbances, it might be argued that the reduced volume of the interstitium rather than net protein catabolism might have contributed to the increased tyrosine concentration found in muscle interstitial fluid of envenomated rats. However, it should be noted that muscle blood flow, as estimated by ethanol technique, did not change after BjV infusion, which suggests that protein metabolism was altered by direct effects of venom on muscle tissue. Despite this evidence, this study can not totally rule out the possibility that microvesSEL damage induced by venom and possibly not detected by ethanol technique may have contributed to the muscle catabolic effect by the oxygen deprivation. These isolated muscles experiments showed that the rate of tyrosine release and incorporation was, respectively, increased and decreased in EDL muscles from BjV-injected rats, suggesting that the muscle interstitial tyrosine increase observed in microdialysis experiments was probably caused by the activation of proteolytic pathways and inhibition of protein synthesis.

The main system involved with the ARF-induced increase in muscle protein breakdown is the UPS.\textsuperscript{46} Also, it has been shown that activation of the UPS is the main cause of muscle protein catabolism induced by fasting, cancer, cachexia, denervation, disuse, excess glucocorticoids, etc.\textsuperscript{11} We further showed that the activity of this proteolytic system is also increased in EDL muscle from rats after \textit{Bothrops} envenomation. As far as we know, this is the first demonstration that the UPS plays a role in the pathogenesis of muscle lesion in a model of snakebite-induced ARF. To further advance in terms of the mechanisms by which BjV increases activity of Ub-proteasome pathway, we measured the content of Ub-protein conjugates 4 hours after venom infusion. The data showed that the process of protein ubiquitination is not affected by the venom. In addition, this study showed that the proteolytic lysosomal system also participates in the loss of proteins in EDL muscles from envenomated rats. In contrast to UPS, the precise contribution of the lysosomal pathway to muscle-wasting disorders is still unclear.\textsuperscript{12} Acidic proteases like cathepsins are relatively nonselective and mostly involved in the degradation of long-lived proteins.\textsuperscript{47} Interestingly, it has been recently shown that lysosomal proteolysis and UPS are coordinately activated in atrophying muscle cells by the same transcription-dependent mechanism involving FoxO3.\textsuperscript{48} Although the cause of proteolytic activation induced by \textit{Bothrops} envenomation cannot be explained on the basis of these data, we speculate that uremia might be involved. The loss of muscle mass in uremia is caused by suppression of anabolic cellular signals and activation of caspase-3 and the UPS.\textsuperscript{9} One factor that increases these proteases in muscles from uremic rats is metabolic acidosis, which could also contribute to the acidification of intracellular pH and stimulates cathepsin activity. The fact that cathepsin B and L content was not altered by venom infusion reinforces this hypothesis. It is also possible that BjV induces a decreased sensitivity of the skeletal muscle to anabolic hormones, such as insulin or insulin growth factors.\textsuperscript{49} Further experiments are needed to clarify these mechanisms and to study whether these alterations on muscle protein metabolism are applicable to other viperid venoms.
In summary, the data indicated that BjV-induced ARF in rats is associated with hemodynamic disturbances and alterations in carbohydrate and protein metabolism, including a rapid and transitory increase in arterial blood pressure, uremia, hyperactaemia, activation of liver glycogenolysis, and a high rate of protein catabolism in skeletal muscle. The loss of proteins in EDL muscles induced by BjV is caused by the reduction of protein synthesis and activation of lysosomal pathway and UPS. These findings may be useful for a better understanding of the clinical picture after snakebites and may bring new perspectives for efficient treatment of muscle-wasting conditions.

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REFERENCES


