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# Sympathetic outflow activates the venom gland of the snake *Bothrops jararaca* by regulating the activation of transcription factors and the synthesis of venom gland proteins

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## SUMMARY

**The venom gland of viperid snakes has a central lumen where the venom produced by secretory cells is stored. When the venom is lost from the gland, the secretory cells are activated and new venom is produced. The production of new venom is triggered by the action of noradrenaline on both $\alpha_1$- and β-adrenoceptors in the venom gland. In this study, we show that venom removal leads to the activation of transcription factors NFκB and AP-1 in the venom gland. In dispersed secretory cells, noradrenaline activated both NFκB and AP-1. Activation of NFκB and AP-1 depended on phospholipase C and protein kinase A. Activation of NFκB also depended on protein kinase C. Isoprenaline activated both NFκB and AP-1, and phenylephrine activated NFκB and later AP-1. We also show that the protein composition of the venom gland changes during the venom production cycle. Striking changes occurred 4 and 7 days after venom removal in female and male snakes, respectively. Reserpine blocks this change, and the administration of $\alpha_1$- and β-adrenoceptor agonists to reserpine-treated snakes largely restores the protein composition of the venom gland. However, the protein composition of the venom from reserpinized snakes treated with $\alpha_1$- or β-adrenoceptor agonists appears normal, judging from SDS-PAGE electrophoresis. A sexual dimorphism in activating transcription factors and activating venom gland was observed. Our data suggest that the release of noradrenaline after biting is necessary to activate the venom gland by regulating the activation of transcription factors and consequently regulating the synthesis of proteins in the venom gland for venom production.**

Key words: sympathetic innervation, transcription factors, protein synthesis, exocrine gland, snake, *Bothrops jararaca*.

## INTRODUCTION

*Bothrops jararaca* (Serpentes, Viperidae) is a Brazilian solenoglyphous venomous snake. Its venom gland is an oral exocrine gland that is related to salivary glands, and has the capacity to secrete toxic proteins (Kochva and Gans, 1970). This venom gland has two important characteristics: the presence of a central lumen where most of the venom produced is stored, and a long venom production cycle that lasts around 30–50 days, with distinct steps which include an active stage after loss of venom from the lumen, and a quiescent stage when the lumen is filled (Kochva, 1960; Kochva, 1987; Mackessy, 1991). Because the activation of the venom gland can be explored step by step, it can be used to elucidate the mechanisms involved in the activation of exocrine glands. Besides, snake venom is a rich source of active molecules for bioprospecting studies and, therefore, understanding how the venom is produced allows us to establish a functional secretory cell line in order to obtain large-scale venom production *in vitro.*

After venom is lost from the lumen of the gland, a cycle of venom production and secretion is initiated, which starts with morphological and biochemical changes of the secretory epithelium. The epithelial cells change their shape from cuboid to columnar, the cisternae of the rough endoplasmatic reticulum expand, and venom is synthesized. The maximal synthetic activity of the secretory cells and the highest mRNA concentrations are observed after 4–8 days. Afterward, the synthetic activity decreases, and venom gradually accumulates in the gland lumen, while the epithelium returns to the quiescent stage (Ben-Shaul et al., 1971; Carneiro et al., 1991; De Lucca et al., 1974; Kochva, 1978; Oron and Bdolah, 1973; Rotenberg et al., 1971). Thus, a complete venom production cycle is much longer than the protein production cycle in mammalian salivary and pancreatic glands (Amsterdam et al., 1969; Jamieson and Palade, 1967a; Jamieson and Palade, 1967b).

We have shown that the noradrenergic innervation present in the venom gland has an essential role in triggering the venom production cycle. Both $\alpha_1$- and β-adrenoceptors are involved in this process (Yamanouye et al., 1997; Yamanouye et al., 2000; Kerchove et al., 2004). The $\alpha_1$-adrenoceptor is desensitized immediately after venom removal, suggesting that noradrenaline is released in the venom gland at this time, and is resensitized 30 days later (Kerchove et al., 2004). The sensitivity of this receptor for noradrenaline and phenylephrine is low (Kerchove et al., 2004). It is coupled to a Gq protein, and triggers the venom production cycle by activating the phosphatidylinositol 4,5-bisphosphate and extracellular signal-regulated kinase (ERK) signalling pathways (Kerchove et al., 2008). Like mammalian adrenoceptors, the β-adrenoceptor is coupled to Gs protein, as its stimulation increases cyclic AMP production, but its sensitivity to drugs differs from that of mammalian adrenoceptors. This receptor seems to become uncoupled from its second messenger system in activated cells (Yamanouye et al., 2000).

Sympathetic outflow stimulates the synthesis of salivary proteins in mammals (Barka et al., 1986; Woon et al., 1993; Ann and Lin, 1997; Ann and Lin, 1998), and sympathetic stimulation with isoprenaline changes the gene expression pattern in the salivary

gland (Ten Hagen et al., 2002). These data led us to the hypothesis that noradrenergic innervation could be important for the synthesis of venom proteins and/or proteins involved in venom production. Thus, the aim of the present study was to determine how noradrenergic innervation triggers the venom production cycle. Here, we show that venom removal and noradrenaline activate the transcription factors NFκB and AP-1. We also show for the first time that noradrenergic innervation is a key activator of this exocrine gland. Noradrenergic stimulation is required for the production of venom gland proteins, unlike in the mammalian salivary gland, where noradrenaline is directly involved in the synthesis of salivary proteins.

## MATERIALS AND METHODS

### Materials

Bovine serum albumin, crystalline reserpine, (–)-arterenol bitartrate, L-phenylephrine hydrochloride, (–)-isoproterenol bitartrate, protease inhibitor cocktail P8340, Kodak MS film, Tris, Hepes, EDTA and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St Louis, MO, USA). NFκB and AP-1 consensus oligonucleotides were purchased from Promega (Madison, WI, USA). T4 polynucleotide kinase was purchased from Invitrogen (Carlsbad, CA, USA). Poly(dI-dC), [$\gamma^{32}$P]-ATP nucleotide and MicroSpin G-25 columns were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). NP40, U-73122, myristoylated cell permeable protein kinase A inhibitor 14-22 amide (PKI), H89 dihydrochloride and staurosporine from *Streptomyces* sp. were purchased from Calbiochem (La Jolla, CA, USA). The protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sodium pentobarbital was purchased from Cristália (São Paulo, SP, Brazil). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

### Animals and venom gland

*Bothrops jararaca* (Wied 1824) adults of both sexes (*N*=88), weighing 150–400 g, were classified by the Laboratory of Herpetology of the Instituto Butantan, and kept in a room under controlled conditions instead of maintained as described by Breno and colleagues (Breno et al., 1990). Snakes were kept without access to food for 40 days before the start of the experiment to make sure that most of the cells in the venom gland were in the quiescent stage. Fasting periods of 1–2 months are common in snakes living in the wild, but fasts can exceed 1 year (Secor and Nagy, 1994). Animal care and procedures used were in accordance with guidelines of the Animal Ethics Committee of the Instituto Butantan and the Biomedical Science Institute of the University of São Paulo.

Snakes were anaesthetized with sodium pentobarbital (30 mg kg$^{-1}$, s.c.) and decapitated, and the venom gland was removed and freed from connective tissue (Yamanouye et al., 2007). To remove the venom, snakes were anaesthetized with sodium pentobarbital (20 mg kg$^{-1}$, s.c.) and the venom was removed manually (Belluomini, 1968).

### Preparation of dispersed cells

Venom glands were dissected from female snakes from which no venom had been removed for at least 40 days in order to obtain cells in a quiescent stage. Quiescent secretory cells were dispersed as previously described (Yamanouye et al., 2000; Kerchove et al., 2004; Yamanouye et al., 2007) and resuspended in Krebs-Hepes solution (composition in mmol l$^{-1}$: NaCl 120; KCl 4; $MgSO_4$ 1.2; $KH_2PO_4$ 1.2; Hepes 15; $CaCl_2$ 2.5; with ascorbic acid 0.01% and glucose 10; pH 7.4) and used immediately.

### Nuclear protein extraction

Nuclear extracts were prepared as described by Rong and Baudry (Rong and Baudry, 1996) with some modifications. Briefly, dissected venom glands were homogenized (tissue homogenizer with Teflon pestle, Thomas Scientific, Swedesboro, NJ, USA) in cold lysis buffer (2.6 µl buffer mg$^{-1}$ of wet tissue, buffer composition: 10 mmol l$^{-1}$ Hepes pH 7.5, 10 mmol l$^{-1}$ KCl, 0.1 mmol l$^{-1}$ EDTA pH 8.0, 10% glycerol, 1.0 mmol l$^{-1}$ DTT, protease inhibitor cocktail dilution 1:100) and incubated on ice for 15 min. A volume of 25 µl of 10% nonidet P-40 was added to a final volume of 400 µl and the mixture was vortexed for 10 s, and centrifuged at 12,000 ***g*** for 2 min at 4°C. The pellet was washed with lysis buffer and recentrifuged at 12,000 ***g*** for 2 min at 4°C. The nuclear pellet was resuspended in nuclear extraction buffer (10 mmol l$^{-1}$ Hepes pH 7.5, 0.5 mmol l$^{-1}$ KCl, 1 mmol l$^{-1}$ EDTA pH 8.0, 10% glycerol, 1.0 mmol l$^{-1}$ DTT, protease inhibitor cocktail dilution 1:100) at 0.7 µl mg$^{-1}$ wet tissue, incubated on ice for 15 min, and then centrifuged at 13,000 ***g*** for 20 min at 4°C. The supernatant containing the nuclear proteins was stored at –70°C until use. The protein concentration of the nuclear extract of venom gland was determined by the method described by Bradford (Bradford, 1976).

To prepare nuclear extracts, $1.5\times10^6$–$3\times10^6$ dispersed quiescent secretory cells were incubated in 400 µl cold lysis buffer and broken by repeated pipetting. The nuclear pellet was resuspended in 100 µl of nuclear extraction buffer. Other procedures were the same as for the nuclear extract of venom gland except the washing step, which was omitted.

### Electrophoretic mobility shift assay

Double-stranded NFκB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′) consensus oligonucleotides were end-labelled with [$\gamma$-$^{32}$P]-ATP (specific activity of 3000 Ci mmol$^{-1}$) in the presence of 10 units T4 polynucleotide kinase (10 min, 37°C). Unincorporated nucleotides were removed by passing the reaction mixture through a Microspin G-25 column. A gel shift assay was performed as described by Staal and colleagues (Staal et al., 1990) with some modifications. A total of 7–15 µg of nuclear extract protein or 15 µl of nuclear extract of secretory cells was incubated for 20 min at room temperature with 2 µl gel shift binding buffer (50 mmol l$^{-1}$ NaCl, 0.2 mmol l$^{-1}$ EDTA pH 8.0, 0.5 mmol l$^{-1}$ DTT, 10% glycerol, 10 mmol l$^{-1}$ Tris-HCl pH 7.5) and 1 µg poly(dI-dC) in a final reaction mix volume of 22 µl. Each sample was then incubated for 30 min at room temperature with 30,000–50,000 c.p.m. of purified $^{32}$P-labelled probe. Protein–DNA complexes were separated by electrophoresis through a 6% non-denaturing acrylamide:bis-acrylamide (37.5:1) gel in Tris-borate/EDTA (TBE buffer 0.5×) at 150 V for 1.5 h at room temperature. The gels were vacuum dried, and exposed to Kodak MS film for 4–7 days at –70°C. Autoradiography films were scanned and analysed densitometrically with MCDI M4 image analysis 3.0 software (Imaging Research, St Catharine, Ontario, Canada). For competition studies, unlabelled NFκB or AP-1 double-strand oligonucleotide was added in molar excess over radiolabelled probe.

### Preparation of extract of the venom gland

Venom gland extracts were prepared based on the protocol described by Gonçalves and colleagues (Gonçalves et al., 1997). Briefly, venom glands from which all venom had been removed from the lumen were cut into 250 µm slices (McIlwain Tissue Chopper, Vibratome Company, O'Fallon, MO, USA) and homogenized (tissue homogenizer, Teflon pestle, Thomas Scientific); 15 g of

homogenate was mixed with 100 ml ice-cold solution containing 0.32 mol $l^{-1}$ sucrose, 1 mmol $l^{-1}$ EDTA, 3 mmol $l^{-1}$ $MgCl_2$ and 1 mmol $l^{-1}$ PMSF. The homogenate was centrifuged at 14,000 ***g*** for 40 min at 4°C. The supernatant containing cytosolic proteins was recovered and stored at –20°C until use.

### Analysis of protein composition

The protein concentration of venom gland extracts and venom was determined by the method of Bradford (Bradford, 1976) using Bio-Rad reagents and bovine serum albumin as a standard. Venom (10 µg protein) or venom gland extract (30 µg protein) was denatured in sample buffer (Laemmli, 1970) for 5 min at 100°C. The proteins were separated by SDS-PAGE (12% or 15%) with the buffer system described by Laemmli (Laemmli, 1970). The proteins were stained with Coomassie Brilliant Blue. The gel was scanned and the density of the bands was quantified with Quantity One software (Bio-Rad).

### Statistical analysis

The results are expressed as means ± s.e.m. of the indicated number of experiments. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons with Graph-Pad Prism 3.0 software (San Diego, CA, USA). A probability of less than 0.05 was considered statistically significant.

### Design of experiments

#### Time course of transcription factor activation after venom removal

Venom glands were obtained from female and male snakes ($N$=5 for each group) from which no venom was removed (quiescent stage) and from snakes that had their venom removed 30, 60 or 120 min before they were killed by decapitation. Nuclear extract of the venom gland was prepared, and the activation of the NFκB and AP-1 transcription factors was analysed by the electrophoretic mobility shift assay as described above.

#### Activation of transcription factors by stimulation of adrenoceptors in venom gland

Nuclear extracts were prepared from dispersed secretory cells obtained from venom glands in the quiescent stage from female snakes ($N$=18). A total of $1.5\times10^6$–$3.0\times10^6$ cells was incubated for 30 or 60 min at 30°C in Krebs-Hepes solution containing noradrenaline (0.1 mmol $l^{-1}$), phenylephrine (0.3 mmol $l^{-1}$) or isoprenaline (0.3 mmol $l^{-1}$) in a final volume of 500 µl. The concentrations used were based on the $EC_{50}$ of these agonists determined by functional studies using microphysiometry (Kerchove et al., 2004; M. B. Zablith and N.Y., unpublished data from our laboratory).

To verify the upstream pathway, cells were incubated at 30°C with inhibitors of phospholipase C (PLC; U73122, 10 µmol $l^{-1}$ for 60 min), protein kinase C (PKC; staurosporine, 100 nmol $l^{-1}$, 30 min), PKA/PKC (H89, 90 µmol $l^{-1}$, 30 min) or protein kinase A (PKA; PKI, 10 µmol $l^{-1}$, 30 min). Noradrenaline (0.1 mmol $l^{-1}$) was then added and the cells were incubated for another 30 min. The activation of transcription factors was analysed by the electrophoretic mobility shift assay described above and normalized to $1\times10^6$ cells. The venom gland of male snakes was not used because their smaller gland yielded fewer secretory cells.

#### Effect of adrenoceptor stimulation on venom composition

The venom of snakes was manually removed to start the venom production cycle. Six snakes were treated with reserpine (20 mg $kg^{-1}$, s.c., 24 h before venom removal, followed by daily injections of 5 mg $kg^{-1}$, s.c. for 15 days). This dose of reserpine completely eliminates noradrenaline from the venom gland (Yamanouye et al., 1997). Some reserpine-treated snakes received phenylephrine (100 mg $kg^{-1}$, s.c.) or isoprenaline (100 mg $kg^{-1}$, s.c.) (Nunez-Burgos et al., 1993) just after the removal of venom. A second sample of venom was collected 15 days after the first collection. The protein composition of the samples was analysed by SDS-PAGE. The first sample was used as a control to reduce effects of inter-individual variation on venom composition (Meier, 1986; Chippaux et al., 1991; Monteiro et al., 1998a; Monteiro et al., 1998b).

#### Time course of changes in venom gland proteins

Venom glands were obtained from female and male snakes in which venom was not removed previously (quiescent stage, $N$=2) and female and male snakes that had their venom removed manually 4, 7 or 15 days (activated gland, $N$=2 for each group) before they were killed by decapitation. The venom gland proteins were separated by SDS-PAGE.

#### Effect of adrenoceptor stimulation on the composition of the venom gland

Four female and four male snakes were treated with reserpine (20 mg $kg^{-1}$, s.c., 24 h before the first venom removal, followed by daily s.c. injections of 5 mg $kg^{-1}$). Some reserpine-treated snakes received phenylephrine (100 mg $kg^{-1}$, s.c.) and isoprenaline (100 mg $kg^{-1}$, s.c.), just after the removal of venom. Female snakes were killed 4 days later, male snakes were killed 7 days later. The venom glands were dissected and the venom gland proteins were separated by SDS-PAGE. Control snakes had their venom removed but were not treated with reserpine, and they were killed 4 days (females) or 7 days (males) later.

## RESULTS

### Venom removal stimulates the activation of NFκB and AP-1 in the venom gland of *Bothrops jararaca*

After venom has been removed, the secretory cells start the production of venom, which is accompanied by changes in the venom gland morphology and rate of protein synthesis (Kochva, 1987). Noradrenaline starts the venom production cycle by stimulating both $\alpha_1$- and β-adrenoceptors (Yamanouye et al., 1997; Yamanouye et al., 2000; Kerchove et al., 2004). We examined whether venom removal could activate transcription factors such as NFκB and AP-1.

The transcription factors NFκB and AP-1 were present in the nuclear extract of the quiescent venom gland. Competition studies using unlabelled NFκB and AP-1 double-strand oligonucleotides showed that binding was specific (Fig. 1A; Fig. 2A).

Venom removal caused the activation of NFκB in the venom gland (Fig. 1A). As shown in Fig. 1B, the activation of NFκB was increased in nuclear extracts of venom gland 30 min (% above baseline level: 33.21±7.16, $N$=5 and 16.43±6.8, $N$=4), 60 min (% above baseline level: 16.21±3.83, $N$=5 and 38.86±9.61, $N$=4) and 120 min (% above baseline level: 12.67±4.8, $N$=5 and 11.54±2.09, $N$=4) after venom removal in female and male snakes, respectively. NFκB activation peaked 30 min after venom removal in female snakes and 60 min after venom removal in male snakes (Fig. 1B).

Venom removal also caused AP-1 activation (Fig. 2A). As shown in Fig. 2B, the activation of AP-1 was increased in nuclear extracts of venom gland 30 min (% above baseline level: 12.51±7.85, $N$=4 and 15.04±7.05, $N$=5), 60 min (% above baseline level: 91.96±25.65, $N$=4 and 56.07±14.96, $N$=5) and 120 min (% above baseline level:

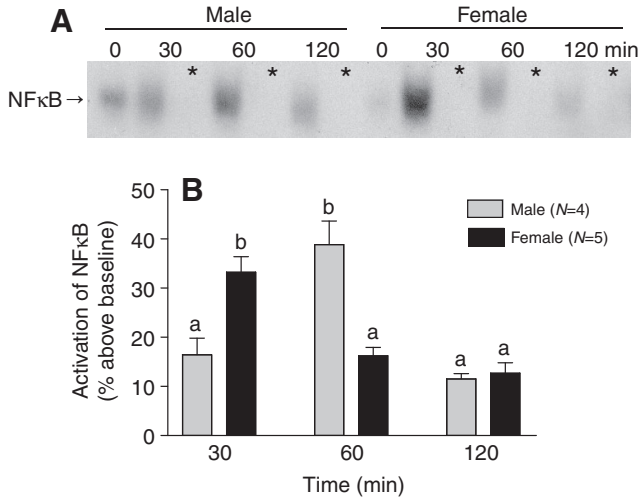


Fig. 1. Time course of NFκB activation in the venom gland of the snake *Bothrops jararaca* after venom removal. (A) Nuclear extracts from venom gland of male and female snakes in quiescent stage (0 min) and 30, 60 and 120 min after venom removal were assayed for the level of NF-κB binding to $^{32}$P end-labelled NFκB oligonucleotide probe by the electrophoretic mobility shift assay (EMSA). *Competition assays were performed using an excess of unlabelled specific NFκB oligonucleotide. (B) Densitometric analysis of NFκB activation above baseline level in EMSA using nuclear extracts from venom glands of male and female snakes 30, 60 and 120 min after venom removal. Note the difference in time of activation between males and females. Statistical analysis of differences between nuclear extracts was performed, and different lowercase letters indicate significant differences (ANOVA, Newman–Keuls, $P<0.05$).

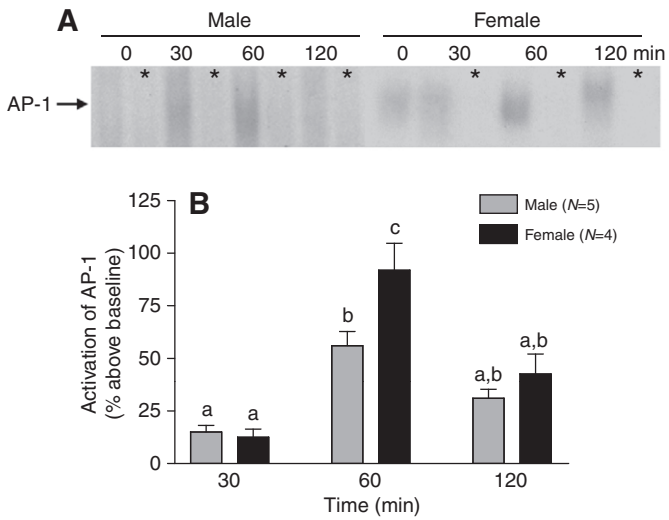


Fig. 2. Time course of AP-1 activation in the venom gland of the snake *Bothrops jararaca* after venom removal. (A) Nuclear extracts from venom gland of male and female snakes in quiescent stage (0 min) and 30, 60 and 120 min after venom removal were assayed for the level of AP-1 binding to $^{32}$P end-labelled AP-1 oligonucleotide probe by EMSA. *Competition assays were performed using an excess of unlabelled specific AP-1 oligonucleotide. (B) Densitometric analysis of AP-1 activation above baseline level in EMSA using nuclear extracts from venom glands of female and male snakes 30, 60 and 120 min after venom removal. Statistical analysis of differences between nuclear extracts was performed, and different lowercase letters indicate significant differences (ANOVA, Newman–Keuls, $P<0.05$).

42.67±18.89, $N$=4 and 31.08±9.67, $N$=5) after venom removal in female and male snakes, respectively. AP-1 activation peaked 60 min after venom removal (Fig. 2B, $P<0.05$) and was more pronounced in female than in male snakes ($P<0.05$).

### Stimulation of $\alpha_1$- and β-adrenoceptors activates NFκB and AP-1 in secretory cells

To examine the role of sympathetic outflow in the activation of transcription factors, we used dispersed secretory cells from quiescent venom glands of female snakes and stimulated them for 30 min with noradrenaline, phenylephrine or isoprenaline. The venom glands of male snakes were not used because they are smaller than female glands and yield few secretory cells.

Noradrenaline (0.1 mmol l$^{-1}$) caused the activation of NFκB (24.60±8.83% above baseline level, $N$=6; Fig. 3B,C). Both the α-agonist phenylephrine (0.3 mmol l$^{-1}$) and the β-agonist isoprenaline (0.3 mmol l$^{-1}$) also stimulated NFκB activation (% above baseline level: 19.31±5.29, $N$=6 and 30.35±4.58, $N$=8, respectively; Fig. 3A,C) in quiescent secretory cells stimulated for 30 min.

Noradrenaline (0.1 mmol l$^{-1}$ for 30 or 60 min) also caused the activation of AP-1 (% above baseline level: 12.01±4.21, $N$=3 and 19.19±5.92, $N$=4, respectively; Fig. 4A–D). Isoprenaline increased

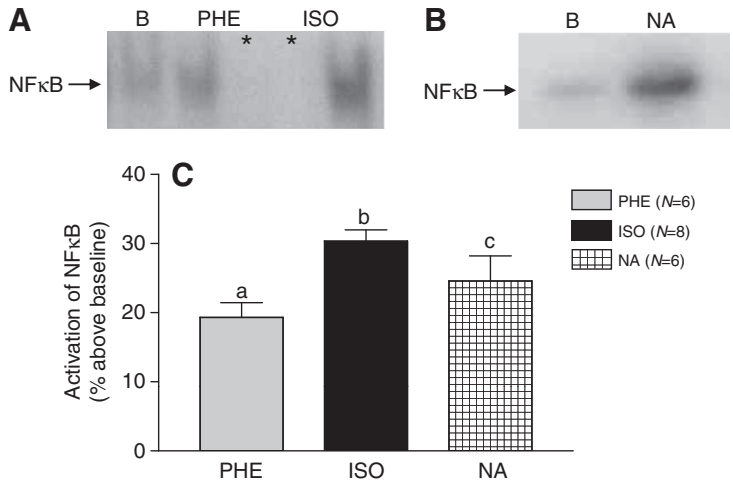


Fig. 3. Stimulation of $\alpha_1$- and β-adrenoceptors activates NFκB in secretory cells of the venom gland of female *Bothrops jararaca*. EMSA using nuclear extracts of quiescent secretory cells (labelled B) and cells incubated for 30 min with (A) phenylephrine (PHE, 0.3 mmol l$^{-1}$), isoprenaline (ISO, 0.3 mmol l$^{-1}$) or (B) noradrenaline (NA, 0.1 mmol l$^{-1}$). *Competition assays were performed using an excess of unlabelled specific NFκB oligonucleotide over radiolabelled probe. (C) Densitometric analysis of NFκB activation in EMSA using nuclear extract of quiescent secretory cells incubated for 30 min with NA, PHE or ISO. Statistical analysis of differences between nuclear extracts was performed, and different lowercase letters indicate significant differences (ANOVA, Newman–Keuls, $P<0.05$).

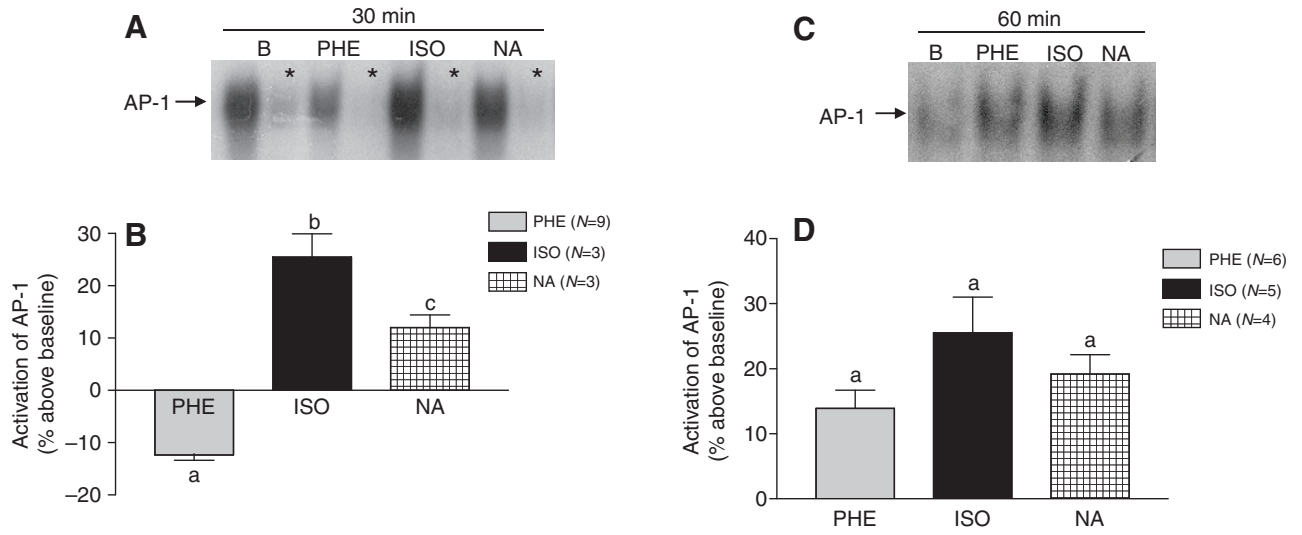


Fig. 4. Stimulation of $\alpha_1$- and β-adrenoceptors regulates AP-1 activation in secretory cells of the venom gland of female *Bothrops jararaca.* (A,C) Nuclear extracts from secretory cells of quiescent venom gland of female snakes (labelled B) or cells incubated with 0.1 mmol $l^{-1}$ NA, 0.3 mmol $l^{-1}$ phenylephrine or 0.3 mmol $l^{-1}$ ISO for 30 or 60 min, respectively. *Competition assays were performed using an excess of unlabelled specific AP-1 oligonucleotide. (B,D) Densitometric analysis of AP-1 activation in EMSA using nuclear extract of quiescent secretory cells incubated for 30 or 60 min with NA, PHE or ISO. Statistical analysis of differences between nuclear extracts was performed, and different lowercase letters indicate significant differences (ANOVA, Newman–Keuls, $P<0.05$).

the activation of AP-1 in quiescent secretory cells stimulated for 30 and 60 min (% above baseline level: 25.49±7.73, $N=3$ and 25.52±12.30, $N=5$, respectively; Fig. 4A–D). However, phenylephrine only increased the activation of AP-1 after 60 min of stimulation (13.93±6.81% above baseline level, $N=6$; Fig. 4C,D). After 30 min of stimulation, phenylephrine decreased the activation of AP-1 (12.67±2.94% below baseline level, $N=9$; Fig. 4A,B).

In order to verify the upstream signalling pathway, we measured the effect of inhibitors of PLC (U73122, 10 μmol $l^{-1}$), PKC (staurosporine, 100 nmol $l^{-1}$), PKC/PKA (H89, 90 μmol $l^{-1}$) and PKA (PKI, 10 μmol $l^{-1}$) on the response to noradrenaline. As shown in Table 1, U73122, staurosporine, H89 and PKI significantly reduced the activation of NFκB by noradrenaline (0.1 mmol $l^{-1}$, 30 min). All inhibitors tested except staurosporine significantly reduced the activation of AP-1. We did not examine upstream signalling pathways activated by 60 min of incubation with noradrenaline because the effect was different from the sum of the adrenergic agonists, suggesting the activation of additional mechanisms.

### Effect of reserpine and stimulation of $\alpha_1$- and β-adrenoceptors on venom composition

To examine whether the stimulation of $\alpha_1$- and β-adrenoceptors affects the composition of the venom, we used reserpine to deplete endogenous catecholamine stores (Yamanouye et al., 1997) and injected $\alpha_1$- or β-adrenoceptor agonist (100 mg $kg^{-1}$ s.c. each) (Nunez-Burgos et al., 1993). Due to inter-individual variation in venom composition (Meier, 1986; Chippaux et al., 1991; Monteiro et al., 1998a; Monteiro et al., 1998b), we used the first sample of venom of the same snake as the control.

As shown in Fig. 5, the venom protein profile of samples collected with a 15 day interval was similar in untreated control snakes (lanes 1 and 2). Reserpine completely abolished venom production, as shown previously (Yamanouye et al., 1997). The administration of isoprenaline to reserpine-treated snakes restored venom production, and the composition of the venom was similar to that of the control sample (lanes 3 and 4). Similarly, phenylephrine restored venom production, and the composition of the venom was like that of the control sample (lanes 5 and 6).

### Activation of the venom gland is accompanied by regulation of levels of specific venom gland protein

To test whether increased venom production after removal of venom is accompanied by changes in the synthesis of proteins of the venom gland, we emptied the venom gland 0, 4, 7 and 15 days into the venom production cycle, and analysed the cytosolic protein profile of the (venom-free) gland extract. In female snakes, bands of approximately 81, 69, 47, 44 and 38 kDa were increased 4 days after venom removal, and bands of approximately 28 and 19 kDa were reduced 7 days after venom removal (Fig. 6A). In male snakes, bands of approximately 81, 69, 57, 54, 47 and 44 kDa were increased 7 days after venom removal and bands of approximately 38, 28 and 17 kDa were reduced, 4 days after venom removal (Fig. 6B). Venom gland composition

Table 1. Upstream signalling of NFκB and AP-1 activation by noradrenaline

| Treatment | NFκB (% above baseline) | AP-1 (% above baseline) |
|---|---|---|
| NA | 13.53±3.03$^a$ (7) | 20.92±8.29$^a$ (5) |
| NA + U73122 | –7.49±2.06$^b$ (7) | –36.63±6.08$^b$ (5) |
| NA + H89 | –3.62±2.87$^c$ (4) | –12.87±11.11$^c$ (4) |
| NA + staurosporine | 6.77±4.26$^c$ (4) | 19.19±9.09$^a$ (4) |
| NA + PKI | 2.62±3.42$^c$ (5) | 8.76±3.53$^d$ (6) |

Cells were incubated with inhibitors of phospholipase C (U73122, 10 μmol $l^{-1}$ for 60 min), protein kinase C (staurosporine, 100 nmol $l^{-1}$, 30 min), protein kinase A/C (H89, 90 μmol $l^{-1}$, 30 min) or protein kinase A (PKI, 10 μmol $l^{-1}$, 30 min) and then incubated with noradrenaline (NA, 0.1 mmol $l^{-1}$) for 30 min at 30°C. Data represent means ± s.e.m. ($N$). Different lowercase letters indicate statistically significant difference ($P<0.05$; ANOVA, Newman–Keuls test).

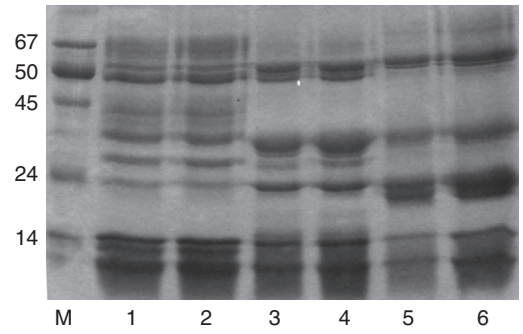


Fig. 5. Effect of stimulation of $\alpha_1$- and β-adrenoceptors on venom composition in snakes treated with reserpine. The venom of snakes was manually removed twice, with a 15 day interval, and 10 μg of venom proteins were separated by SDS-PAGE (12%). Lanes 1, 3 and 5 show first samples, collected before treatment, and lanes 2, 4 and 6 show samples taken at 15 days from the same snakes: lane 2 untreated (no reserpine), lane 4 treated with reserpine plus isoprenaline, and lane 6 treated with reserpine and phenylephrine. Figure is representative of three independent experiments. M, marker lane (kDa).

returned to that of the quiescent stage 15 days after venom removal (Fig. 6A,B).

### Stimulation of $\alpha_1$- and β-adrenoceptors in reserpine-treated snakes is accompanied by regulation of levels of specific venom gland proteins

We investigated the role of $\alpha_1$- and β-adrenoceptors in the changes that occur in the composition of the venom gland during the venom production cycle by treating the snakes with reserpine and giving $\alpha_1$- plus β-adrenoceptor agonists. Venom-free venom glands of female snakes were examined 4 days after venom removal, and of male snakes 7 days after venom removal, as the maximal changes in venom gland composition occurred at these times. Fig. 7 shows that reserpine caused large changes in the cytosolic protein profile of the venom gland. The protein profile of reserpine-treated glands appeared similar to that of quiescent glands (compare Fig. 6A with Fig. 7A, and Fig. 6B with Fig. 7B), with a reduced density of bands of approximately 81, 69, 47, 44, 41 and 38 kDa in female snakes, and a reduced density of bands of 81, 69, 57, 54, 47 and 44 kDa in male snakes. In female snakes, the administration of isoprenaline with phenylephrine (100 mg $kg^{-1}$ of each, s.c.) at the time of venom removal resulted in a pattern that appeared similar to that observed in controls not treated with reserpine (Fig. 7A). However, in male snakes, adrenoceptor stimulation only partly reversed the effect of reserpine (bands of 57 and 54 kDa; Fig. 7B).

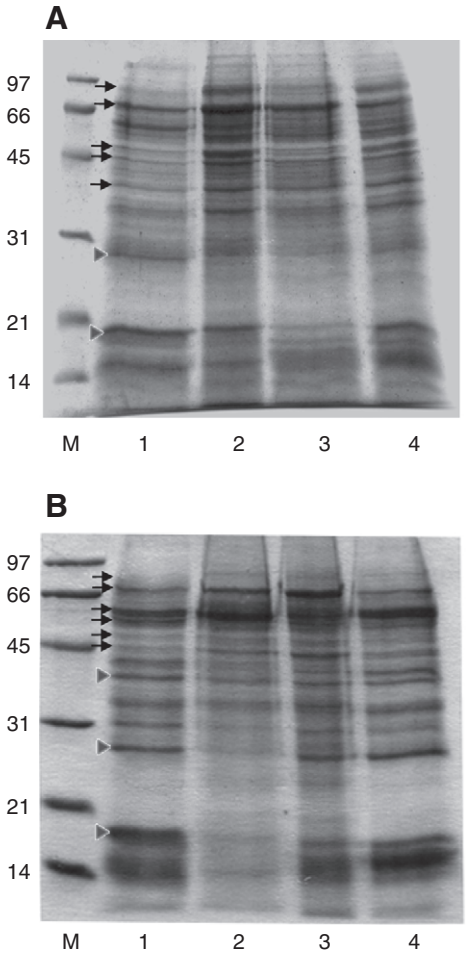


Fig. 6. Protein composition of venom gland tissue of *Bothrops jararaca* at different times after venom removal. (A) SDS-PAGE (12%) gel of female venom gland tissue (30 μg of protein) in the quiescent stage, and 4, 7 and 15 days after removal of venom (lanes 1, 2, 3 and 4, respectively). Four days after venom removal, bands of 81, 69, 47, 44 and 38 kDa were increased (arrows), and bands of 28 and 19 kDa were reduced 7 days after venom removal (arrowheads), when compared with quiescent cells. Figure is representative of five independent experiments. (B) SDS-PAGE (15%) gel of male venom gland tissue (30 μg of protein) in the quiescent stage, and 4, 7 and 15 days after removal of venom (lanes 1, 2, 3 and 4, respectively). Arrows indicate bands with increased (81, 69, 57, 54, 47 and 44 kDa) density, 7 days after venom removal and arrowheads indicate bands with reduced (38, 28 and 17 kDa) density, 4 days after venom removal, when compared with quiescent cells. Figure is representative of two independent experiments. M, marker lane (kDa).

## DISCUSSION

Our results demonstrate that the transcription factors NFκB and AP-1 are present in the snake venom gland, and that they are activated by the removal of venom. Similar transcription factor activation is induced by noradrenaline, which is normally released after the loss of venom from the venom gland. In addition, we showed that the removal of venom induces changes in the protein composition of the venom gland tissue. This effect depends on the release of noradrenaline, because it is blocked by reserpine, and noradrenaline is necessary for the production of new venom. However, noradrenaline does not change venom protein synthesis. Therefore, it seems that the production of the complex mixture of venom proteins is determined by a fixed programme of biochemical changes in the venom-producing cells. The start of this programme is triggered by noradrenergic stimulation, and both $\alpha_1$- and β-adrenoceptors participate in this process.

Activation of transcription factors is a complex process and involves multiple intracellular signalling factors, including kinases such as PKC and PKA (Sheng et al., 1991; Karin, 1995; McBride and Nemer, 1998). In *Bothrops jararaca* venom gland, stimulation of $\alpha_1$-adrenoceptors in quiescent secretory cells increases total inositol phosphate, mobilizes calcium from intracellular stores, and activates PKC and ERK (Kerchove et al., 2008), and stimulation of β-adrenoceptors in quiescent secretory cells selectively increases cAMP production and cytosolic calcium concentration by activating voltage-operated and receptor-operated $Ca^{2+}$ channels (Yamanouye et al., 2000; M. B. Zablith and N.Y., unpublished data from our

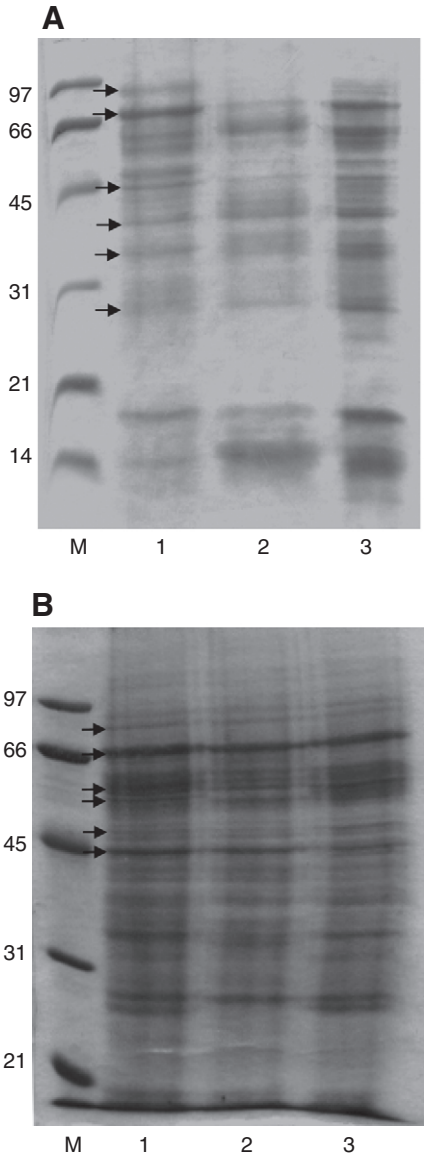


Fig. 7. Effect of reserpine and stimulation of $\alpha_1$- and β-adrenoceptors on the protein composition of venom gland tissue after venom removal. (A) SDS-PAGE (12%) gel shows female venom gland tissue (30 μg of protein) 4 days after removal of venom from a snake not treated with any drug (lane 1), and snakes treated with reserpine (lane 2), and reserpine plus isoprenaline plus phenylephrine (lane 3). Arrows indicate bands of 81, 69, 47, 44, 41 and 38 kDa that were reduced by reserpine. Figure is representative of two independent experiments. (B) SDS-PAGE (15%) gel shows male venom gland tissue (30 μg of protein) 7 days after removal of venom from a snake not treated with any drug (lane 1), and snakes treated with reserpine (lane 2), and reserpine plus isoprenaline plus phenylephrine (lane 3). Arrows indicate bands of 81, 69, 57, 54, 47 and 44 kDa that were reduced by reserpine. Figure is representative of two independent experiments. M, marker lane (kDa).

laboratory). These messengers may interact with additional proteins and subsequently activate transcription factors, contributing to regulation of gene expression.

Venom removal or noradrenaline can activate NFκB and AP-1 in the venom gland. NFκB activation was stimulated by both $\alpha_1$- and β-adrenoceptor agonists. AP-1 activation seemed to depend mostly on β-adrenoceptors; the delayed AP-1 activation (after an initial inhibition) induced by $\alpha_1$-adrenoceptor stimulation may be a subsequent event. Inhibitors of PLC, PKC and PKA reduced the activation of NFκB by noradrenaline, whereas AP-1 activation was reduced by PLC and PKA inhibitors, but not by inhibition of PKC.

Our finding that the stimulation of $\alpha_1$- and β-adrenoceptors causes activation of NFκB and AP-1 in the venom gland is in line with several studies that show that the stimulation of α- and β-adrenoceptors induces c-*fos* expression in a variety of cells, including cardiac myocytes, vascular smooth muscle cells, neuroblastoma cells, brown adipocytes and hepatocytes (Iwaki et al., 1990; Shilling et al., 1991; Okazaki et al., 1994; Thonberg et al., 1994; Garcia-Sáinz and Alcántara-Hernández, 1996; Im et al., 1998; Taimor et al., 2004). All α-adrenoceptor subtypes were able to induce the expression of c-*fos* and c-*jun*, and this effect seems to be mediated by PKC, but $\alpha_1$-adrenoceptor subtypes vary in the efficacy of this induction (García-Saínz et al., 1998). Activation of NFκB can be induced by both α- and β-adrenoceptors (Meldrun et al., 1997; Aksoy et al., 2001; Chandrasekar et al., 2004; Lymperopoulos et al., 2006).

The administration of reserpine blocked the production of new venom (Yamanouye et al., 1997). The protein profile of the reserpine-treated gland suggests that reserpine caused the gland to remain in the quiescent stage. Reserpine has been used to examine the role of sympathetic innervation of the salivary gland in rats, but it causes irreversible morphological and functional changes in the salivary glands, similar to those observed in patients with cystic fibrosis (Martinez et al., 1975a; Martinez et al., 1975b; Watson et al., 1984; Johnson, 1988). In the snake, the effect of reserpine does not seem to be due to damage to the gland, as adrenoceptor stimulation reverses the effect of reserpine (Yamanouye et al., 1997). The reversible effect of reserpine may be due to the capacity of the venom gland to maintain a quiescent stage. In contrast, the salivary gland is constantly in the activated stage.

The similarity of the effects of $\alpha_1$- and β-adrenoceptor stimulation on the composition of the venom is remarkable, as these receptors are coupled to different G proteins, release different second messengers, and activate different transcription factors. Nevertheless, both induced the production of the full gamut of venom proteins.

The administration of $\alpha_1$- and β-adrenoceptor agonists to reserpine-treated snakes restores venom gland activation, suggesting that noradrenaline is important in triggering the cells to start the venom production programme; in other words, to activate the secretory cells to produce venom. It is known that sympathetic innervation plays an important role in stimulating the synthesis and secretion of salivary proteins (Mehansho and Carlson, 1983; Barka et al., 1986; Woon et al., 1993; Ann and Lin, 1997; Ann and Lin, 1998). In contrast, in the venom gland of the snake, noradrenergic stimulation seems to regulate the production of components of the machinery for venom production, rather than directly stimulating the synthesis of venom toxins. In accordance with our results, Nunez-Burgos and colleagues (Nunez-Burgos et al., 1993) have also shown that chronic administration of isoproterenol to snakes alters the composition of proteins of the quiescent venom gland.

The importance of β-adrenoceptors in the synthesis of salivary gland proteins is well known. Chronic administration of isoprenaline in mammals leads to hyperplasia and hypertrophy of acinar cells and changes in the content of many proteins in the salivary gland by increasing DNA and RNA synthesis (Barka, 1965; Brown-Grant, 1961; Schneyer, 1962; Selye et al., 1961; Vugman and Hand, 1995; Woon et al., 1993). Using microarray technology, Ten Hagen and colleagues (Ten Hagen et al., 2002) showed early changes in gene

expression in the murine parotid gland exposed to isoprenaline. Proteins in several functional classes were affected and could be related to the hyperplasia or hypertrophy found before. However, there are no studies that have examined the role of salivary gland α-adrenoceptors in protein synthesis.

Our data also showed a sexual dimorphism in the time of maximal activation of NFκB, and the intensity of activation of AP-1 in the venom gland. The difference in AP-1 activity may be partially caused by differences in NFκB activation, which is known to affect AP-1 activity (Fujioka et al., 2004; Krappmann et al., 2004). These differences in the time course and level of the transcription factors may contribute to the faster activation of the venom gland in female than in male snakes (see Figs 6 and 7), and may also contribute to the sexual dimorphism in activity and protein composition of *Bothrops jararaca* venom reported by Menezes and colleagues (Menezes et al., 2006). Sex-based variation also occurs among bradykinin-potentiating peptides present in the venom (Pimenta et al., 2007). It is interesting to note that gene expression in the human parotid gland is also gender dependent (Srivastava et al., 2008).

In conclusion, we showed that noradrenaline released after venom removal promotes the activation of transcription factors, by stimulating both $\alpha_1$- and β-adrenoceptors, and as a consequence changes the synthesis of proteins of the venom gland, and that these proteins are involved in activating the gland. Further studies are needed to identify the proteins involved in venom gland activation. We also showed sex-based differences in venom gland activation. It is important to point out that the venom gland of Viperidae snake could be an attractive model to study physiological regulation of protein synthesis in exocrine glands as, in contrast to salivary glands, the venom gland can assume distinct quiescent and activated stages. It is interesting to note that the sympathetic nervous system is not involved in the synthesis of toxin proteins as seen in salivary proteins of salivary glands.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AP-1 | activator protein 1 |
| ERK | extracellular signal-regulated kinase |
| NFκB | nuclear factor κ B |
| PKA | protein kinase A |
| PKC | protein kinse C |
| PKI | protein kinase A inhibitor 14–22 amide, cell permeable, myristoylated |
| PLC | phospholipase C |
| s.c. | subcutaneously |

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