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# Pharmacological characterisation of arthritis induced by *Bothrops jararaca* venom in rabbits: a positive cross talk between bradykinin, nitric oxide and prostaglandin $E_2$

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BACKGROUND: Our previous results showed that nitric oxide (NO) and bradykinin (BK) mediate the arthritis induced by ***Bothrops jararaca*** venom (BjV) in rabbits. In this study, we investigated the contribution of each receptor of BK as well as the inter-relationship between NO and eicosanoids in BjV-induced arthritis.

***Methods***: The arthritis was induced in rabbits with 16 μg of BjV injected intra-articularly. Prostaglandin $E_2$ ($PGE_2$), thromboxane $B_2$ ($TxB_2$), leukotriene $B_4$ ($LTB_4$) (radioimmunoassay) and nitrite/nitrate concentrations ($NO_2/NO_3$) (Griess reaction) were evaluated in the synovial fluid 4 h later. The animals were prior treated with NO synthase inhibitor (L-NAME; 20 mg/kg/day for 14 days), the B2 antagonist of BK (HOE-140) and the B1 antagonist of BK (des-Arg$^9$[Leu$^8$]-bradykinin), both at a dose of 0.3 mg/kg, 30 min prior to the venom injection.

***Results***: Data show that L-NAME and HOE-140 treatment were equally able to reduce $PGE_2$ and $NO_2/NO_3$ levels without interfering with $TxB_2$ and $LTB_4$ production. On the contrary, the B1 antagonist of BK inhibited $TxB_2$ and $LTB_4$ production, and did not alter $PGE_2$ and NO metabolites levels in the inflamed joint.

***Discussions***: The results presented clarify the contribution of the kinin system, mainly through the B2 receptor, to the local inflammatory response induced by BjV, as well as its positive interaction with $PGE_2$ and NO production.

Key words: *Bothrops jararaca* venom, Inflammatory reaction, Arthritis, Bradykinin, Nitric oxide, Eicosanoids

## Introduction

Endogenous nitric oxide (NO) has been described as a pro-inflammatory molecule showing interactions with cytokines[1–3] and inflammatory products of the cyclooxygenase pathway.[4–10] Studies have shown that NO increases interleukin-1β and eicosanoid products, which may result in an exacerbated inflammatory response.[2,3] In addition, inhibition of NO biosynthesis by an L-arginine antagonist reduced prostaglandin $E_2$ ($PGE_2$) production in different models of inflammatory reactions *in vivo*.[11–13]

Additionally, bradykinin (BK) and NO have been implicated on eicosanoids and cytokine secretions in several experimental inflammatory processes.[8,12,13–15] BK acts on the acid arachidonic metabolism via both phospholipase $A_2$[16] and phospholipase C[17] activation. Also, BK is known to release NO from various cells, including polymorphonuclear[18] and endothelial cells at the site of injury.[19]

The acute inflammatory reaction induced by *Bothrops jararaca* venom (BjV) is determined by oedema and leukocyte influx.[20–22] These effects are mediated by histamine, serotonine, products of arachidonic acid metabolism, platelet-activating factor and factors derived from complement system.[20–22] The eicosanoids contribution is relevant to oedema formation and leukocyte recruitment during BjV-induced pleurisy and air-pouch inflammatory reaction.[21,22] Recently, to determine the participation of kinins and NO in the local inflammatory reaction induced by BjV, we developed an experimental model of arthritis by injecting the venom intra-articularly. The findings that B2 BK antagonist (HOE-140) and NO synthase inhibitor (L-NAME) treatment greatly reduced the cellular influx and protein leakage in the arthritis induced by BjV clearly implicate kinins and NO in this phenomenon.[23]

Since eicosanoids, BK and NO are important mediators involved in the inflammatory reaction evoked by BjV, and since there is strong evidence regarding the interaction between these mediators on different models of the inflammatory process, the aim of this study was to evaluate the participation of each receptor of BK in NO and eicosanoid production in the acute inflammatory response evoked by BjV.

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## Material and methods

### Induction of arthritis

The Animal Ethics Committee of COBEA (Brazilian College of Experimental Animals) approved all experimental procedures, performed on animals in accordance with procedures set by The Universities Federation for Animals Welfare. The arthritis was induced in the knee joint of Male New Zealand White rabbits (2 kg) by the injection of BjV provided by the Laboratory of Herpetology, Butantan Institute, Brazil (16 μg diluted in 0.5 ml of saline). The venom was filtered in sterilising membrane (ester cellulose filter, 0.22 μm pore size; Millipore, São Paolo, Brazil) before joint injection. The contralateral joint was injected with the same amount of bovine serum albumin (BSA) (Sigma, St Louis, MO, USA). The amount of protein present in BjV or BSA solution was spectrophotometrically assessed and the equivalent protein concentration was injected in each joint. Four hours after intra-articular challenge, the animal was anaesthetised with a mixture of xylazine (5 mg/kg; Bayer, São Paolo, Brazil) associated with ketamine (50 mg/kg; Parke Davis, São Paolo, Brazil) by intramuscular injection and killed by intravenous injection of 2.0 ml of 20% KCl solution. Immediately after the sacrifice, 2 ml of saline containing ethylenediamine tetraacetic acid (1 mg/ml) was injected into the knee joint. Synovial fluid was aspirated, the joint was opened and the remainder of the synovial fluid was recovered and stored at –70°C.

### Pharmacological treatments

A group of animals were randomly treated with L-NAME (Sigma), 20 mg/kg/day mixed with drinking water, administered for 2 weeks prior to the induction of arthritis. Treatments with 0.3 mg/kg of B1 or B2 BK antagonists des-Arg$^9$[Leu$^8$]-bradykinin (Sigma) and HOE-140 (Hoechst, Frankfurt, Germany), respectively, were performed subcutaneously, 30 min before the venom injection. Control animals received an equivalent volume of sterile saline by the same route.

### Determination of total $NO_2$·/$NO_3$· levels in the synovial fluid

The concentration of the total amount of nitrate and nitrite ($NO_2$·/$NO_3$·) anions (stable NO breakdown products *in vivo*) present in the synovial fluid samples collected 4 h after venom injection was determined by Griess reaction. The absorbance was monitored at 546 nm.

### Determination of eicosanoids in the synovial fluid

$PGE_2$, thromboxane $B_2$ ($TxB_2$), leukotriene $B_4$ ($LTB_4$) levels were assayed in the synovial fluid collected 4 h after venom injection using commercial kits (NEN Life Science products, Boston, MA, USA) as previously described.[24,25] The competitive binding radioimmunoassays were performed in polypropylene tubes with the reagents diluted in phosphate-buffered saline (pH 6.8) with gelatine and thimerosal. One hundred microlitres each of unknown samples, of tracers ($^{125}$I-$PGE_2$ and $^{125}$I-$TxB_2$ or $^3$H-$LTB_4$), and of rabbit antiserum to each eicosanoids were combined and incubated overnight at 8°C for $LTB_4$ determination, and were then incubated for 2 h at 25°C in a water bath for $PGE_2$ and $TxB_2$ determinations. Unbound radiolabelled $PGE_2$ and $TxB_2$ was removed by the addition of 250 μl of donkey anti-rabbit serum coated onto magnetisable polymer particles, and $LTB_4$ was removed by the addition of 250 μl of 2% charcoal suspension coated with 0.4% dextran to the tubes. After centrifugation, the residual bound activity was measured in each tube by counting $^{125}$I-$PGE_2$ and $^{125}$I-$TxB_2$ in a γ-scintillation counter, and $^3$H-$LTB_4$ in a β-scintillation counter. Cross-reactivities of the antiserum with other eicosanoids were below 0.05%. The assay sensitivities were 1.25–160 pg/tube ($PGE_2$), and 1.6–200 pg/tube ($LTB_4$).

### Statistics

Results are expressed as mean ± standard error of mean. Results were analysed by Student's *t*-test or by repeated-measures analysis of variance. When appropriate, the data were analysed by the Newman-Keuls test. The chosen level of significance was 0.05.

## Results

### Eicosanoids and $NO_2$·/$NO_3$ levels in the synovial fluid

The synovial fluid collected from animals pre-treated with L-NAME, HOE-140 and des-Arg$^9$[Leu$^8$]-bradykinin and intra-articularly injected with venom, and that from respective controls, was extracted to $PGE_2$, $LTB_4$ and $TxB_2$. The determinations were performed by radioimmunoassay. $NO_2$·/$NO_3$ levels in diluted joint wash were also determined by Griess reaction. Figure 1A shows the $PGE_2$ level measured in the synovial fluid obtained 4 h after the BjV. Prior treatment of the rabbits with L-NAME or B2 antagonist of BK HOE-140 significantly reduced it when compared with values obtained in samples from animals, which did not receive pharmacological treatments. It is important to note that des-Arg$^9$l[eu$^8$]-bradykinin treatment did not interfere with the production of $PGE_2$. On the contrary, levels of $LTB_4$ and $TxB_2$ (represented in Fig. 1B and Fig. 1C, respectively) exhibited a different pattern of alteration. They were not altered after treatment with L-NAME or HOE-140, but they were greatly reduced

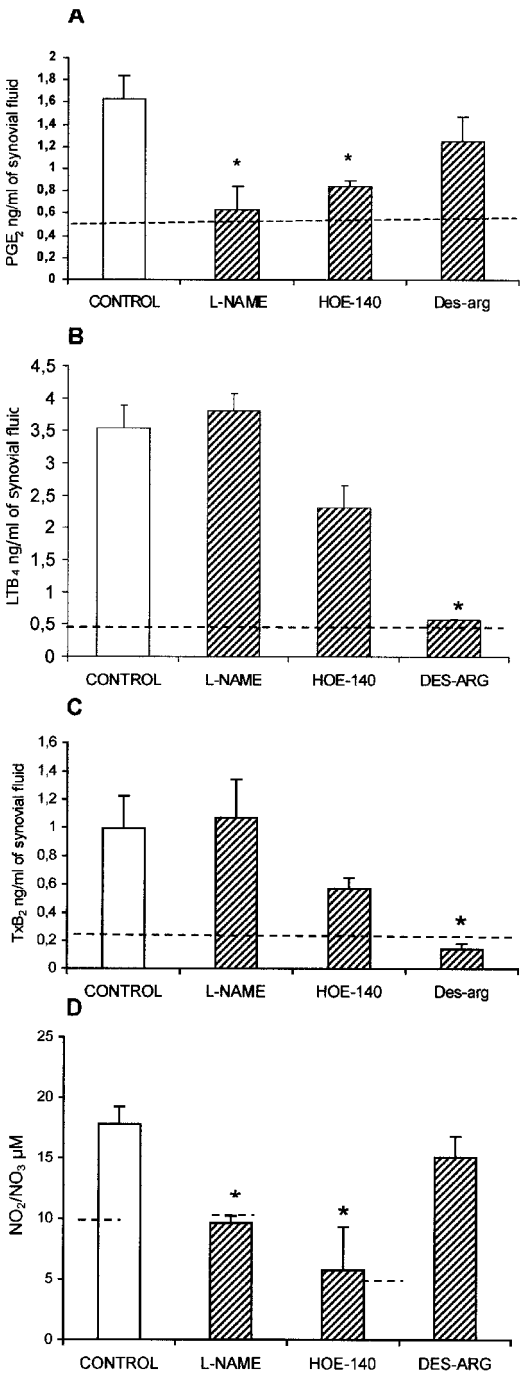


FIG. 1. Effects of L-NAME, HOE-140 and des-Arg$^9$[leu$^8$]-bradykinin treatments on local eicosanoid and NO production 4 h after intra-articular injection of BjV. (A) $PGE_2$ levels, (B) $LTB_4$ levels (C) $TxB_2$ levels and (D) $NO_2$·/$NO_3$· levels following treatments with L-NAME ($n$ = 5), HOE-140 ($n$ = 4) and des-Arg$^9$[Leu$^8$]-bradykinin ($n$ = 5). Open column, the level of the eicosanoid in control animals ($n$ = 6). Dotted line, the values of each eicosanoid in the BSA-injected joint. Each column expresses data as the mean (±s.e.m.). *$p$ < 0.05 by comparison with control animals.

after pre-treatment with the B1 antagonist of BK des-Arg$^9$[Leu$^8$]-bradykinin. Also, values observed in samples from B1 antagonist-treated animals were significantly lower than that obtained in samples from control animals. At the fourth hour after the injection of BSA, the contralateral joint exhibited low levels of the eicosanoids, similar to the observed in the saline-injected joint (data not shown).[12,26]

Similar to the observed pattern of alteration promoted in $PGE_2$ production, the level of the total amount of the NO metabolites ($NO_2$·/$NO_3$·; Fig. 1D) was significantly reduced in the synovial fluid collected from L-NAME-treated and HOE-140-treated animals when compared with the level observed in control animals. Differently, the treatment with the B1 antagonist of BK, des-Arg$^9$[Leu$^8$]-bradykinin, did not affect NO production in the joint, which exhibited values of $NO_2$·/$NO_3$ obtained 4 h after BjV injection equivalent to those found in control animals.

## Discussion

The data presented in this paper regarding BjV local effects corroborate the positive cross-talk between BK, NO and the cyclooxygenase (COX) product $PGE_2$ described in the inflammatory reaction induced by other different agents,[8,12,13] and additionally show the main participation of the B2 BK receptor in this axis. This last observation is in accordance with recent experimental studies in rats that demonstrated HOE-140 treatment reduced the oedema induced by local injection of *Bothrops lanceolatus* snake venom and abolished the hyperalgesic effect of BjV.[27,28] Additionally, the employment of specific receptor antagonists and enzymatic inhibitors in experimental studies has demonstrated the participation of BK receptors and COX metabolites on the inflammatory process evoked by BjV through measurement of its symptomatic effects such as oedema,[20,21,29] leukocyte recruitment[21,22,29] and pain.[28] However, in the experimental model employed in this study, it was possible to establish the link between each mediator involved in the kinin/NO/COX axis at the inflammatory site after pharmacological treatments.

We have clearly demonstrated the contribution of the kinin system, via the B2 receptor, on NO production, with subsequent stimulation of $PGE_2$ secretion. Also, our results showed that the B2 receptor and NO do not participate in $LTB_4$ and $TXB_2$ secretions, which were affected by inhibition of the B1 receptor. These conclusions are demonstrated by the following observations: (1) HOE-140 and L-NAME treatments equally abolished the inflammatory reaction,[23] $PGE_2$ and $NO_2$/$NO_3$ levels without interfering with $LTB_4$ or $TxB_2$ production; and (2) alternatively, des-Arg$^9$[Leu$^8$]-bradykinin treatment did not alter the $PGE_2$ and NO metabolites, and greatly reduced $LTB_4$ and $TxB_2$ secretions. It is important to note that our previous results showed that the NO synthase inhibitor (L-NAME) and the B2 receptor antagonist of BK (HOE-140) treatments promoted a great reduction of the leukocyte influx (90% of the control animals) to the articular cavity.[23] These data associated with the

verified low levels of $LTB_4$ in the joint, suggest the participation of other(s) chemoattractant(s) in this phase of BjV inflammatory reaction. Recent experimental studies have demonstrated that *Bothrops* snake venoms activate the complement system, with a consequent contribution of its metabolites on leukocyte recruitment.[22,30] The participation of the complement system, BK and NO has been described in several models of inflammation.[31,32] The possibility that the complement system participates in or may be affected by BK/NO cascade in the bothropic envenomation will be further investigated. In addition, the treatment with the B1 receptor antagonist of BK, which promoted reduction of $LTB_4$, was less effective than HOE-140, promoting 40% of reduction in the cellular influx to the inflamed area (data not shown).

In conclusion, our results suggest that the NO generation in arthritis induced by BjV is produced mainly after BK stimulation through B2 receptors. Also, the endogenous release of NO enhances COX activity with further increase of $PGE_2$ production, demonstrating the inter-relation between the kinins system with NO and $PGE_2$ in the BjV inflammatory process.

This paper, together our previous results,[23] would be useful to pharmacological interventions to modulate the imminent inflammatory response evoked by BjV, which is not neutralised by specific antivenom.

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