

THE aim of the present study was to investigate the interrelationship of the kinin system, nitric oxide and eicosanoids in the acute phase of antigen-induced arthritis (AIA) in rabbits. The arthritis was induced in immunized rabbits and the following parameters were evaluated 24 hours later: leukocyte influx (total and differential white cell count), vascular permeability (Evans's blue method), and synovial PMN cell infiltrate. PGE₂ and LTB₄ (radioimmunoassay) levels were quantified in the synovial fluid. The animals were pre-treated with 20 mg/kg/day during 14 days with L-NAME or D-NAME and/or Enalapril (0.12 mg/kg/day–14 days), and/or the B2 antagonist of Bradykinin HOE 140 (0.9 mg/kg). Our results showed that L-NAME was effective in the prevention of AIA with reduction of all inflammatory parameters analyzed. Enalapril partially reverted the L-NAME anti-inflammatory effects. The simultaneous treatment with HOE 140 abolished this reversion and returned the inflammatory parameters to the levels observed in L-NAME treated animals. Our results suggest that pressoric alterations induced by L-NAME could not account for all its anti-inflammatory action in this model of experimental arthritis. Additionally the contribution of the kinin system in AIA was characterized as well as its interaction with eicosanoids and nitric oxide.

Key words: Experimental arthritis, Nitric oxide, Eicosanoids, Bradykinin

Interrelationship of the kinin system, nitric oxide and eicosanoids in the antigen-induced arthritis in rabbits

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Introduction

Rheumatoid arthritis is an autoimmune disease in which chronic inflammation of the synovial lining cells produce pain, swelling and progressive erosion of the synovial joints. Dumonde & Glynn¹ first described antigen-induced arthritis (AIA) in rabbits. AIA evolves as a progressive immunological arthritis, which in histopathological terms closely resembles rheumatoid arthritis in man. The acute phase of AIA is a typical Arthus reaction which occurs in the joint with complement activation and polymorphonuclear (PMN) leukocyte migration.²

Nitric oxide (NO) is a very small and ubiquitous molecule synthesized from L-arginine by nitric oxide synthases (NOS). NO is produced in the joint by chondrocytes,^{3–6} synoviocytes^{6,7} and osteoblasts.⁶ It has been involved in inflammatory reactions with both pro and anti-inflammatory properties. Endogenous release of NO could act as anti-inflammatory through the reduction of leukocyte adhesion,^{8,9} and inhibition of synthesis of cyclo-oxygenase products such as prostaglandin E₂ (PGE₂), thromboxane B₂ (TxB₂) and interleukin-6 (IL-6).¹⁰ Endogenous NO

has also been described as a pro-inflammatory molecule showing interactions with cytokines^{11–13} and inflammatory products of the cyclo-oxygenase pathway.^{14–20} Studies have shown that NO increases interleukin-1 β (IL-1) and eicosanoid products which may result in the exacerbation of the inflammatory response.^{12,13} The participation of NO in intra-articular inflammatory process has been fully described in arthritic patients.^{21,22} NOS inhibitors have been widely used in studies of NO influence in the inflammatory process. The administration of NOS inhibitors in the experimental arthritis in rats was followed by reduction in the intra-articular accumulation of leukocytes, joint erosion, and paw swelling as well as histopathological abnormalities.^{23–27} Inhibition of NO biosynthesis by L-arginine antagonism reduced PGE₂ production by chondrocytes³ without effect upon the increased production of PGE₂ by synoviocytes stimulated with IL-1.⁷ Moreover, endogenous release of NO enhances macrophage cyclooxygenase (COX) activity and increases the production of pro-inflammatory prostaglandins.^{13,28} Several studies suggest that NO participates in the inflammatory response and joint destruction. Slices of rabbit

articular cartilage synthesized large quantities of NO following stimulation with human recombinant IL-1 β . Treatment of cartilage fragments with NOS inhibitor decreased NO synthesis stimulated by IL-1 β and restored proteoglycan synthesis.²⁹ We previously verified that chronic treatment of arthritic rabbits with L-NAME evoked reduction of the cellular influx and protein leakage to the articular cavity. Additionally the synovial fluid of these animals showed reduction of PGE₂, IL1 and NO₂/NO₃ levels.³⁰ Chronic administration of NOS inhibitors causes vasoconstriction with potential reduction in cell migration. The contribution of this pressoric alteration evoked by chronic treatment with L-NAME to its anti-inflammatory action as well as the possible links between NO and other mediators of inflammatory reaction have so far been poorly studied in the articular environment. Thus, in the present study we analyzed the leukocyte migration, protein leakage, synovium polymorphonuclear cell infiltrate and eicosanoids production in the synovial fluid of knee joints of rabbits with AIA treated with NOS inhibitor, Enalapril and the B2 antagonist of Bradykinin HOE 140.

Material and methods

Induction of arthritis

The Animal Ethics Committee of COBEA (Brazilian College of Experimental Animals) has approved all experimental procedures performed on animals in accordance with procedures set by UFAW (The Universities Federation for Animals Welfare). Male New Zealand White rabbits were sensitized with 5 mg of methylated bovine serum albumin (mBSA – Sigma) in 1 ml Freund's complete adjuvant (Gibco) and 1 ml of sterile saline through injections at subcutaneous and muscular sites in the suprascapular and gluteal regions, respectively. Seven days after the immunization the animal was once a week boosted with intradermal injection of 1 mg/ml of mBSA. Then, cutaneous Arthus reaction characterized by central necrosis was observed 24 h later. Simultaneously, the serum antibody titres against mBSA were quantified by immunodiffusion. The animals used in the experiment were those who appropriately responded to the second cutaneous challenge and have had anti-mBSA titres over 1/8. Seven days after the third booster, arthritis was induced in the knee joint by the injection of 0.5 ml of a sterile solution of mBSA (2 mg/ml) into the articular cavity. The contralateral joint was injected with saline. Twenty four hours after intra-articular challenge the animal was anaesthetized with a mixture of xylazine (5 mg/kg) associated with ketamine (50 mg/kg) by intramuscular injection and after that it was killed by intravenous injection of 2.0 ml of KCl 20%

Treatments

A group of animals was randomly treated with L-NAME (Sigma), a competitive NOS inhibitor, or its inactive form D-NAME (Sigma). The dose for both treatments was of 20 mg/kg/day mixed with drinking water. Drugs were administered for 2 weeks prior to the induction of arthritis, and started simultaneously with the second booster. Another group of animals was randomly treated with Enalapril, an angiotensin converting enzyme (ACE), which produces blood pressure decrease. The treatment with Enalapril started simultaneously with L-NAME and the dose of the drug was 0.12 mg/kg/day mixed with drinking water. The B2 antagonist of Bradykinin HOE 140 (D-Arg-[Hyp³, Thr⁵, Dtic⁵, Oic⁸]-bradykinin) was injected subcutaneously in a dose of 0.3 mg/kg every 8 hours after the arthritis induction.

Sampling of synovial fluid

Immediately after the sacrifice 2 ml of saline containing EDTA (1 mg/ml) was injected into the knee joint. Synovial fluid was aspirated, the joint was opened and the remainder of the synovial fluid as well as synovial membrane was recovered. Total and differential leukocyte count was done in a Neubauer chamber under light microscopy. For the differential white cell count smears were prepared from a cell pellet and stained with Giemsa. The synovial fluid was stored at -70°C for eicosanoids determination.

Assessment of vascular permeability response with Evans's blue dye

Before mBSA injection to induce arthritis the animals received iv. 20 mg/kg of Evans's blue dye in a 2.5% saline solution. The dye combines with the proteins of the plasma giving rise to a tagged macromolecule that passes the endothelial barrier only to a negligible extent under normal conditions, but it is deposited in the tissues under circumstances of increased vascular permeability. The synovial fluid was centrifuged and the optical density colorimetrically assessed at 630 nm. The concentration of dye bound to protein in the synovial fluid was estimated from a standard graph recording the optical density of serial dilution of a known sample of Evans's blue in NaCl. Results are expressed as μ g of protein/ml of synovial fluid.

Synovial membrane histology

After sampling the synovial fluid and opening the articular cavity, the synovial membrane was excised and sections were stained with hematoxylin and eosin for evaluation of PMN cell infiltrate under light microscopy. The synovium PMN cell infiltrate was quantified as absent or rare, mild, or severe by an observer blinded to the experimental groups.

Assay of eicosanoids

PGE₂ and LTB₄ levels were assayed in the synovial fluid using commercial kits (Amersham, UK) as previously described.^{31,32} The radioimmunoassays were performed in polypropylene tubes with the reagents diluted in phosphate-buffered saline with gelatin and thimerosal. One hundred microliters each of unknown samples, tracers (I¹²⁵ PGE₂ or H³ LTB₄), and rabbit antiserum to each eicosanoid were combined and incubated overnight at 8°C for LTB₄ determination and incubated for 2 hours at 25°C in a water bath for PGE₂ determination. Unbound radiolabeled PGE₂ was removed by the addition of 250 µl of donkey anti-rabbit serum coated onto magnetizable polymer particles, and LTB₄ 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 I¹²⁵ PGE₂ in a gamma scintillation counter and H³ LTB₄ in a beta scintillation counter. Cross reactivities of the antiserum with other eicosanoids were below 0.05%. The assay sensitivities were 1.25 to 160 pg/tube (PGE₂), and 1.6 to 200 pg/tube (LTB₄).

Statistics

Results are expressed as mean ± s.e.m. The results were analyzed by Student's *t* test or by repeated measured ANOVA and compared with student Newman Keuls test. The chosen level of significance was 0.05.

Results

Leukocyte influx and vascular permeability in the synovial fluid

To investigate the contribution of pressoric alteration to the anti-inflammatory properties of chronic L-NAME treatment, leukocyte influx and protein leakage to the articular cavity was quantified in the synovial fluid collected from animals treated with L-NAME or D-NAME with or without Enalapril. Synovial fluid cells and vascular permeability were measured 24 hours after articular challenge with mBSA. Panel A (Fig. 1) shows the effect of L-NAME compared with control animals in the reduction of cellular migration to the inflamed area. The reduced migration occurs mainly in the number of polymorphonuclear (PMN) leukocytes, the central cell in the acute phase of the inflammatory reaction, although mononuclear (MN) cell migration was also affected. The simultaneous administration of Enalapril with L-NAME partially restores this parameter. The cellular influx to the articular cavity observed in animals treated with Enalapril alone was similar to that observed in the animals treated with L-NAME plus Enalapril. The synovial fluid of the control joint, injected with saline exhibited only resident cells (dotted line). Leukocyte

counts in the articular cavity of D-NAME treated animals did not differ from the leukocyte count in control animals, which drank only water³⁰.

The vascular permeability shown in panel B was quantified by Evans's blue method. Our results showed a significant decrease in protein leakage to the articular cavity in the synovial fluid collected from L-NAME treated animals. The knee joint injected with saline showed low levels of dye bound to protein. Similar findings in the protein leakage to the articular cavity were observed in both D-NAME treated animals and those which drank only water. The protein leakage analyzed in the joint fluid of animals treated with Enalapril or Enalapril plus L-NAME did not differ from the control animals. In all groups evaluated the synovial membrane PMN cell infiltrate showed a similar pattern to the PMN infiltrate observed in the synovial fluid (data not shown). In a previous paper we showed that the synovial membrane of animals receiving mBSA intra-articularly had intense PMN cell infiltrate at 24 hours. Treatment with the NOS inhibitor L-NAME, prior to the induction of arthritis, greatly reduced the cell infiltration in the synovial membrane 24 hours after the induction of arthritis.³⁰ Fig. 2, Panels A and B, shows the inflammatory parameters analyzed in animals simultaneously treated with the B2 antagonist of Bradykinin HOE 140. The cellular influx and the protein leakage analyzed in the synovial fluid of these animals were remarkably reduced when compared with the same parameters observed in animals without HOE 140 treatment. It is worth noting that the HOE treatment drastically reduced the inflammatory parameters measured in the synovial fluid of L-NAME plus Enalapril treated animals. These results show the importance of the kinin system in the development of AIA in rabbits.

Eicosanoids levels in the synovial fluid

The synovial fluid of animals treated with L-NAME, D-NAME, Enalapril and HOE140 was collected, and the eicosanoids PGE₂ and LTB₄ assayed by radioimmunoassay. The results are shown in Figs 3 and 4 respectively. The PGE₂ level was significantly reduced with L-NAME treatment, in accordance with our previous results.³⁰ The simultaneous treatment with Enalapril completely reverted this inhibition. The treatment of animals with HOE 140 drastically reduced the level of PGE₂ in all groups evaluated. It is important to note that the same result even occurred in the group of animals treated only with Enalapril plus HOE 140. The level of LTB₄ shown in Fig. 4 exhibited a quite different pattern of alteration. Although reduced after treatment with L-NAME alone or combined with Enalapril, the level of LTB₄ was reverted to that observed in control animals when the treatment included HOE 140. Treatment with D-NAME did not affect the synovial fluid levels of eicosanoids

when compared with the results obtained in animals which drank only water.³⁰

Discussion

Our previous results clearly implicate nitric oxide in the IL-1 induced PGE₂ production in the synovial fluid of acute arthritis in rabbits.³⁰ In this study we also demonstrate that treatment with the NOS inhibitor L-NAME was associated with a reduction in some signs of acute articular inflammation in rabbits such as vascular permeability and synovial membrane PMN cell infiltrate. In the acute phase of AIA we noticed a reduction in the number of leukocytes coming to the inflamed area. This finding is consistent with the study of Belenky *et al.*,³³ who found that NOS inhibitors attenuate chemotaxis of unstimulated and primed PMN leukocytes. Kaplan *et al.*,³⁴ using peripheral human neutrophils incubated with the NOS

inhibitor, LNMA, demonstrated a reduction of leukocyte chemotaxis to FMLP. This inhibition could be overcome if L-arginine or dibutyl cGMP were added together with the LNMA. These studies point out the importance of NO in the direction of cell movement. Our results clearly show that under inflammatory challenge the migratory response of PMN cells and the synovium PMN cell infiltrate were reduced in animals treated with L-NAME. This suggests that the endothelial lining of vessels within the rabbit synovium may be an unusual microvascular network. We hypothesized that chronic treatment with an NOS inhibitor promotes vascular pressoric alteration that could account for its anti-inflammatory action. The simultaneous treatment of these animals with Enalapril, an ACE inhibitor, restored the cellular influx, protein leakage and eicosanoids production almost to the normal range observed in control arthritic joints. BK induces increase in the blood flow of the rabbit

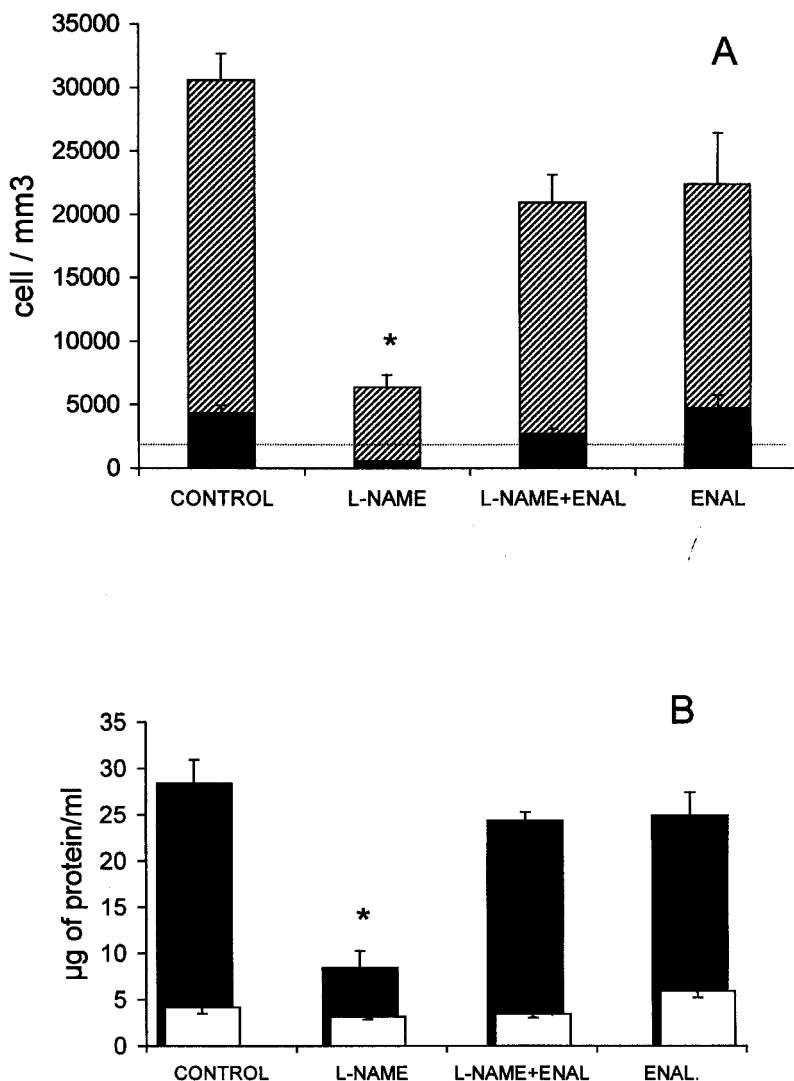


FIG. 1. Panel A: Total and differential cell counts (polymorphonuclear = ▨; mononuclear = ■), and Panel B: Vascular permeability (BSA injected joint = black columns; Saline injected = white columns) assessed in the joint fluid of rabbits with 24 hours of antigen-induced arthritis. The animals were treated before induction of arthritis with L-NAME (n = 16), Enalapril (n = 11), L-NAME+Enalapril (n = 10) and compared with control animals (n = 20). The results expressed mean ± s.e.m.*<0.05.

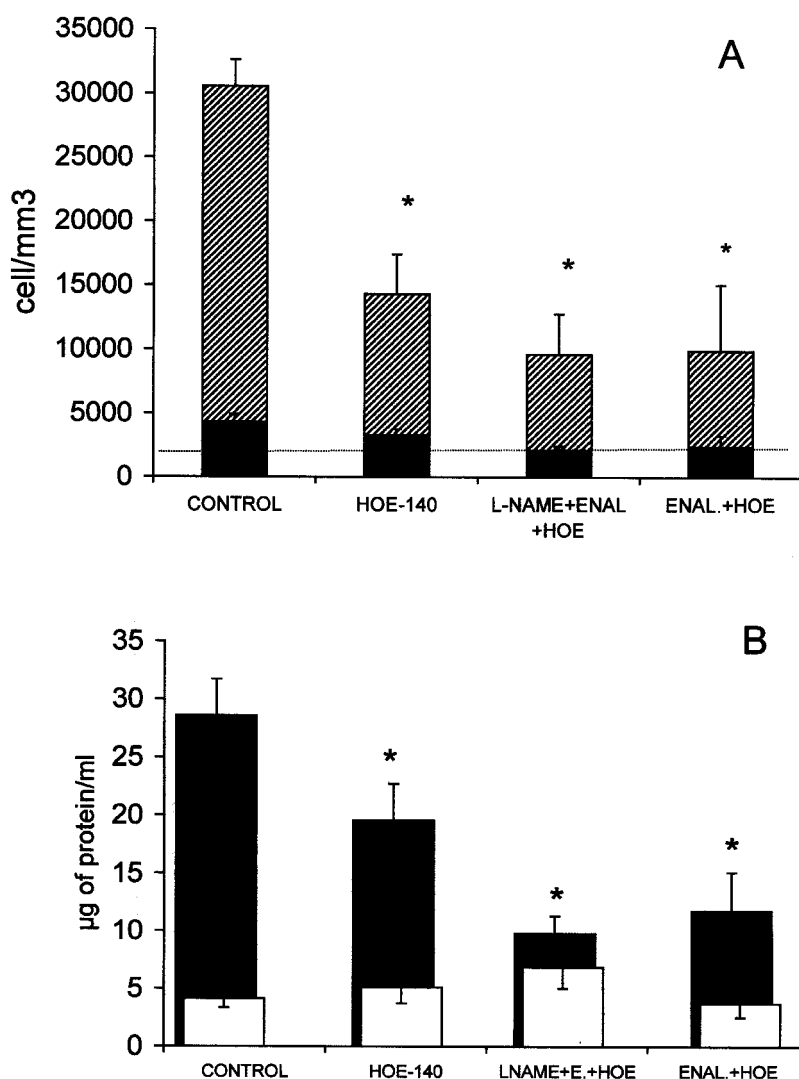


FIG. 2. Panel A: Total and differential cell counts (polymorphonuclear = ▨; mononuclear = ■), and Panel B: Vascular permeability (BSA injected joint = black column; Saline injected = white column) assessed in the joint fluid of rabbits with 24 hours of antigen-induced arthritis. The animals were treated before induction of arthritis with HOE 140 ($n = 4$), Enalapril + HOE ($n = 4$), L-NAME + Enalapril + HOE ($n = 4$). The results expressed mean \pm s.e.m. * <0.05 , compared with mean of 20 control animals.

synovium via activation of B2 receptors.³⁵ Then, we also analyzed the participation of the kinin system through co-administration of a B2 antagonist of BK HOE 140 to the animals, before and during the arthritis development. This treatment promoted remarkable reduction of cellular influx, protein leakage and PGE₂ level in the synovial fluid. It is worth noting that concomitant administration of HOE 140 completely abolished the increase of the inflammatory parameters promoted by Enalapril to L-NAME treated animals.

NO appears to be generated by the vascular endothelial cells via constitutive NO synthase stimulated by inflammatory mediators like histamine, 5 hydroxytryptamine and Bradykinin or by the inducible enzyme carried to the inflammatory site by PMN cells.³⁶ The magnitude in the reduction of the inflammatory parameters observed with HOE 140

treatment suggests that NO generation in experimental arthritic joints follows BK stimulation of B2 receptors. The results obtained in all animals treated with the B2 antagonist of Bradykinin irrespective of other treatments were similar to the values observed in animals treated only with L-NAME and included the level of NO₂/NO₃ (data not shown). The only exception was in the level of LTB₄ measured in the experimental joints of HOE treated animals, which result is compared with those obtained without HOE treatment. This finding suggests a possible inhibitory action of kinin system products in the lipoxygenase pathway.

LTB₄ would be considered a potent chemotactic in the acute phase of inflammatory response.³⁷ We have recently found that LTB₄ levels decreased in the synovial fluid of untreated animals at 24 hours of arthritis when compared with LTB₄ levels found in

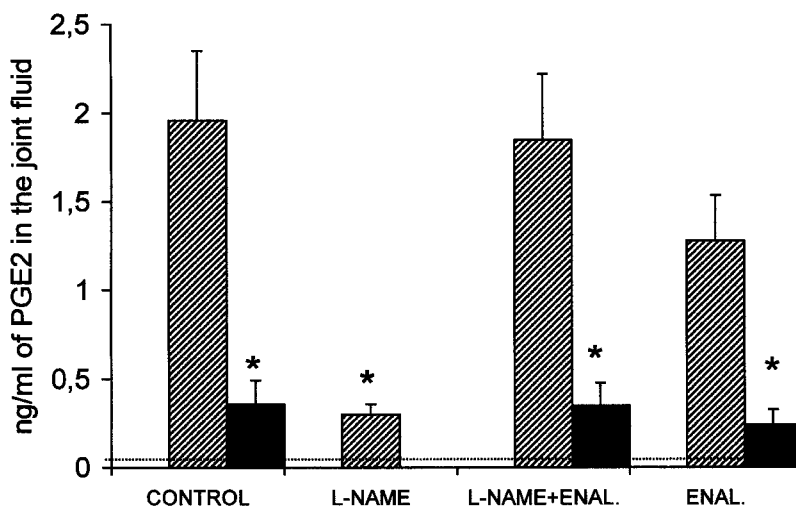


FIG. 3. The striped column shows the PGE₂ levels in the synovial fluid of animals treated with Enalapril (n = 7), L-NAME + Enalapril (n = 7) and control animals (n = 10). The black column shows means of four animals submitted to the same treatments and simultaneously injected with HOE 140. The results expressed mean ± s.e.m. * < 0.05 compared with groups without HOE 140 treatment.

animals at 4 hours of arthritis (LTB₄ 4th h = 2.48 ± 0.16 ng/ml; LTB₄ 24th h = 0.95 ± 0.14 ng/ml).³⁸ The reduced levels of LTB₄ in animals treated only with Enalapril corroborate previous results in arthritic patients treated for arterial hypertension with ACE inhibitors.^{39,40} The reduced levels of LTB₄ and the impaired migration of leukocytes to the articular cavity in L-NAME treated animals suggest that another mediator apart, LTB₄ possibly can be involved in the cell attraction to the articular cavity in this phase of AIA development.

The interactions between PGE₂ IL-1 and NO synthesis have been related in many studies.^{3,4,5,12,20,41} These studies show that the endogenous release of NO enhances COX activity with further increase of PGE₂. High concentrations of PGE₂ inhibited NOS expression and decreased NO synthesis.¹⁴ When NO production was blocked by NOS inhibitors both NO and PGE₂ simultaneously decreased.^{12,16-19,25,28,42} NOS inhibitors also block cytokine induced release

of PGE.^{11,13} In these *in vitro* experiments NO seems to be one of the most important signals in the activation of cyclo-oxygenase to produce PGE₂. We previously found that inhibition of NO production with L-arginine antagonist L-NAME significantly reduced IL-1β and PGE₂ levels in the synovial fluid, with simultaneous reduction of total NO₂/NO₃ and suppression of AIA.³⁰ In this study we verified that treatment with an ACE inhibitor, Enalapril, was efficient in the reversion of inflammatory parameters in the synovial fluid induced by NOS inhibition. This effect may not be in consequence of arterial pressure normalization but due to Bradykinin accumulation at the site of injury and was completely blocked by administration of BK antagonist HOE 140.

Together our results show that the pressoric mechanism acting in chronic administration of L-NAME did not account for all anti-inflammatory properties of NOS inhibitors in the development of

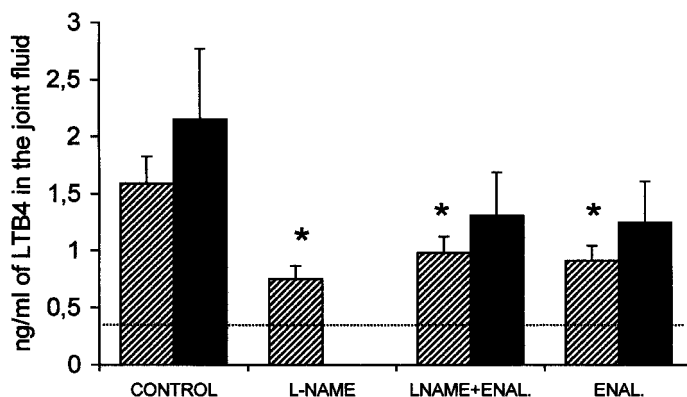


FIG. 4. The striped column shows the LTB₄ levels in the synovial fluid of animals treated with Enalapril (n = 7), L-NAME + Enalapril (n = 7) and control animals (n = 10). The black column shows means of four animals submitted to the same treatment and simultaneously injected with HOE 140. The results expressed mean ± s.e.m. * < 0.05 compared with control animals.

AIA in rabbits. Additionally we demonstrate the participation of Bradykinin in the acute phase of antigen-induced arthritis in rabbits as well as the interrelationship of the kinin system with NO and eicosanoids production in the arthritic joint.

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