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Effects of NOS Inhibitors on Arthritis and Arthritic Pain in Rats

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Among the arthritis symptoms, chronic pain is the most serious, and it can profoundly affect the quality of human life. Unfortunately, the mechanism of development in arthritis and arthritic pain has not yet been precisely elucidated. Accumulating evidence indicates that nitric oxide (NO) plays a pivotal role in nociceptive processing in the spinal cord. However, the modulation mechanism of NO in the peripheral site of arthritis and arthritic pain has not been clarified. Therefore, I determined in the present study which nitric oxide synthase (NOS) was involved in the induction of arthritis and arthritic pain. Monoarthritis was induced by intra-articular injection of carrageenan (2%, 50 μl) into rats, and resulted in the reduction of weight load on the injected leg, increase of knee joint diameter and inflammatory response. Pre-treatment of rats with L-N6-(1-iminoethyl)-lysine (L-NIL, 500 μg in 50 μl), an inhibitor of inducible NOS (iNOS), partially prevented the induction of pain-related behavior and partially reduced inflammatory response in the synovial membrane in the knee joint. These results suggest that iNOS in the knee joint may play an important role in the induction of pain-related behavior and inflammation, and that NO produced by iNOS may be associated with nociceptive signaling in the peripheral site.

Key Words: Nitric oxide (NO), Arthritis, Arthritic pain, Nitric oxide syntase (NOS)

INTRODUCTION

Nitric oxide (NO) is a gaseous mediator that seems to play a role in local circuit nociceptive processing in the spinal cord. It is produced by three major nitric oxide synthase (NOS) isoforms. These are distinguished by their Ca^{2+}/calmodulin dependence and by whether the enzyme is expressed constitutively (constitutive nitric oxide synthase; cNOS) or is induced following exposure to cytokines or endotoxin (inducible nitric oxide synthase; iNOS or type II) (Zimmermann, 2001). Constitutive NOS comprises neuronal NOS (nNOS or type I) which is found in neurons and endothelial NOS (eNOS or type III) which is found in endothelial cells and some central nervous system (CNS) neurons, and transiently produces small amounts of NO that are important in both extracellular and intracellular signalling (Bredt and Snyder, 1992; Moncada et al., 1991). Inducible NO is induced by iNOS which is found in many cell types such as vascular smooth muscle cells, endothelial cells, hepatocytes, macrophages, neutrophils, chondrocytes, synoviocytes and some CNS glial cells (Murphy, 2000; Charles et al., 1993; Palmer et al., 1993; Stadler et al., 1991; Stefanovic-Racic et al., 1992, 1993) in response to inflammatory and immunologic stimuli. iNOS generates much larger quantities of NO over longer periods of time. NO generated by macrophages activated by cytokines and endotoxin contributes to their cytotoxic and cytostatic properties against target cells (Moncada et al., 1991). Although NO generated by constitutive NOS appears to be beneficial in many physiological processes, an excess NO generated by iNOS has been implicated in the pathogenesis of various inflammatory and immunologically mediated diseases, such as graft-vs-host disease (Langrehr et al., 1991), diabetes (Kolb et al., 1991; Corbett er al., 1991), viral infections (Zheng et al., 1993) and arthritis (Farrell et al., 1992; Ialenti et al., 1993).

The administration of a selective inhibitor of the inducible form of NOS can result in dose-dependent inhibition of thermal hyperalgesia which is produced by intraplantar injection of carrageenan (Meller et al., 1994). The upregulation of nNOS in the spinal cord following injection of complete Freund's adjuvant into right tibio-tarsal joint in rat hind paw (Infante et al., 2007) and intraplantar space (Chu et al., 2005) suggested that NO plays an important role in the central mechanism of hyperalgesia, following peripheral inflammation. In addition, it has been shown that mono-arthritic pain is highly sensitive to N^G-nitro-L-arginine methyl ester(L-NAME; a non-selective NOS inhibitor), suggesting involvement of NOS activation in the spinal cord (Laurido et al., 2003) There is also an evidence from a chronic microdialysis analysis that NO is involved in the development of allodynia and thermal hyperalgesia and central sensitization after peripheral inflammation (Yang et al., 1996).

In the adjuvant arthritis model, NO has been shown to be involved in the development of inflammation (Cannon et al., 1996). Intra-articular injection of L-NAME completely reverses heat hyperalgesia and prevents further increase in joint swelling and temperature observed in the model of acute arthritis in which kaolin and carrageenan are injected into the knee joint (Lawand et al., 1997). Most studies of NO and NOS in adjuvant arthritis have focused on the pathologic changes in peripheral tissue, such as the

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knee joint (Connor et al., 1995; Evans, 1995) and paws (Stefanovic-Racic et al., 1994). iNOS, which is related to the inflammation, has also been mentioned to be involved in peripheral pathologic changes. However, there is no direct evidence to show which NOS is involved in the induction of arthritic pain. In this study, the effects of nNOS and iNOS inhibitors on the generation of arthritic pain and inflammatory response in synovial membrane of knee joint tissue were investigated.

METHODS

Subject

Sprague-Dawley male rats (200~300 g; purchased from Sam, Korea) were housed in groups of five in a temperature-controlled room (22~25°C) illuminated from 07:00 to 19:00 hour. Food and water were available ad libitum. However, water was deprived for 12~24 hours before each test day to reinforce the rat in the testing device with water.

Induction of arthritis in rats

To induce arthritis in the experimental rat, lamda-carrageenan (2%, 50 μl, suspended in sterile saline) was injected into the knee joint cavity of the right hind leg of the animal under enflurane anesthesia (0.5~2%). To be sure that the carrageenan injection induced arthritis, the diameters of right and left knee joints before and after the carrageenan injection were measured. The knee joint diameter, defined as the distance between the lateral and medial collateral ligament regions, was measured with calipers while the joint was held in extension. The measurements of joint diameters were made immediately after each weight load test session.

Weight bearing test

In the inflamed joint, weight bearing generates pain perception which is the major symptom in arthritis. Therefore, I utilized a convenient method and apparatus developed by Min et al. (2001) to measure a pain-related behavior (Min et al., 2001). The weight bearing tests were performed 1 day before and 1~4 hours after the injection of carrageenan for pain-related behavior.

Drug treatment

To determine which NOS is involved in the induction of arthritis and arthritic pain, a selective iNOS inhibitor L-NIL hydrochloride (L-NIL; Tocris, Eillisvile, MO, USA) and a selective nNOS inhibitor 7-Nitroindazole (7-Nitro; Biomol research Laboratories Inc., PA, USA, 500 ug in 50 ul) were used. The selection of doses in the present study was based on preliminary experiments. L-NIL and 7-Nitro were dissolved in saline and 4% dimethylsulfoxide (DMSO), respectively. L-NIL or 7-Nitro was injected intra-articularly 5~6 min before carrageenan injection. Control rats were injected with the vehicle.

Histological study

To investigate the effects of NOS inhibitors on inflammation, the inflammatory response of joint tissue at 4 hours after carrageenan injection was examined. Under excessive urethane anesthesia (2.5 g/kg), synovial tissues with patella were removed, conserving the capsule and attached muscles to minimize the loss of synovial membrane. The samples were fixed overnight in 10% formalin, dehydrated through a series of ethanol, and embedded in paraffin wax. Five μm thick sections were cut on a microtome, and stained with hematoxylin-eosin (HE). The acute inflammatory change was quantified histologically by the number of polymorphonuclear cells in the synovial membrane. Ten consecutive high-power fields were captured and counted.

Data analysis

The measured values of weight load and knee joint diameter were normalized; the weight load value measured on a given day was expressed in percent of body weight on that day, and the diameter of the arthritic joint was expressed in percent of the control joint (i.e., left hind leg joint) diameter. Repeated-measures ANOVA test was used to compare the variance among data obtained from a given experimental group at different time points and among data from different experimental groups at a corresponding time point. Post-hoc comparison tests (Scheffe test) were further performed to identify the source(s) of the variance. $p<0.05$ was considered significant.

RESULTS

Effects of intra-articular injection of different NOS inhibitors on pain-related behavior

The carrageenan injection into the knee joint cavity resulted in a significant reduction of weight load on the injected leg and also a significant increase in the knee joint diameter in the control group (data not shown). The

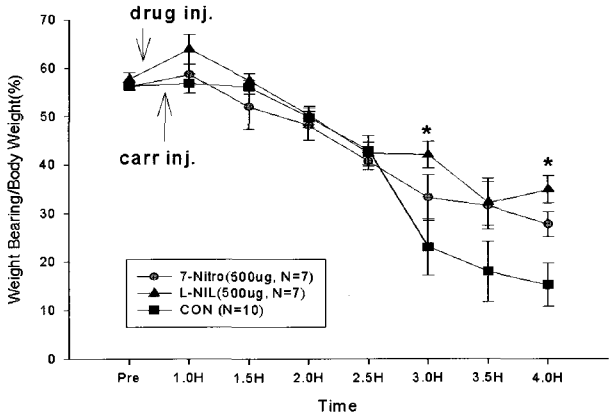

Fig. 1. Time courses of changes produced by intra-articular application of 7-Nitro (nNOS inhibitor), and L-NIL (iNOS inhibitor) on the pain-related behavior induced by carrageenan injection. 7-Nitro (500 μg in 50 μl) or L-NIL (500 μg in 50 μl) was administered 5 min before injection of 2 % carrageenan. Pre-treatment of rats with 7-Nitro partially prevented pain-related behavior induced by carrageenan injection. *$p < 0.05$ compared to Vehicle + Carrageenan-treated rats (Scheffe post hoc test). drug: 7-Nitro, L-NIL or CON (vehicle control), carr: carrageenan. All data are expressed as means ± SEM.

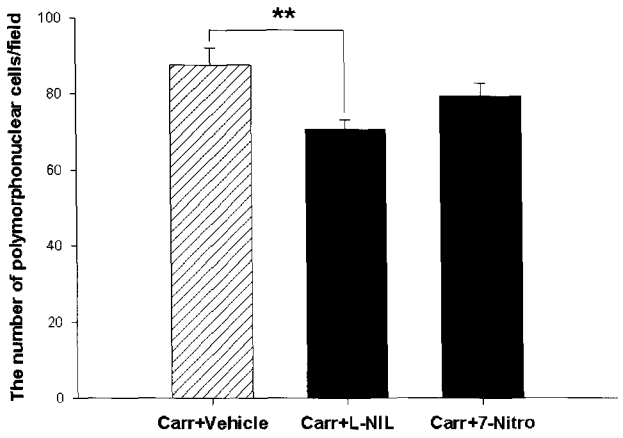

Fig. 2. Bar graphs showing the mean number of polymorphonuclear cells in synovial membrane. The acute inflammatory change was quantified histologically by the number of polymorphonuclear cells in the synovial membrane. Preemptive treatment with intra-articular injection of L-NIL (500 μg in 50 μl) significantly prevented the increase of polymorphonuclear cells in synovial membrane following the injection of 2 % carrageenan. However, nNOS inhibitor 7-Nitro did not block the increase of the number of polymorphonuclear cells induced by carrageenan injection. The number of animal in each group was five. **p $<$ 0.01 compared to vehicle + carrageenan-treated rats (Scheffe post hoc test), carr: carrageenan. All data are expressed as means ± SEM.

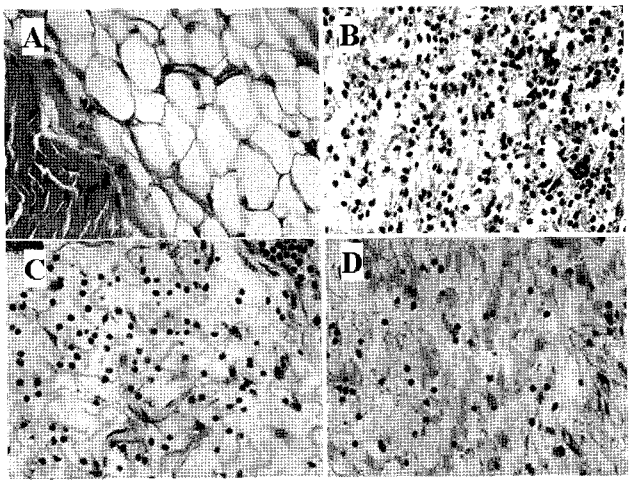

Fig. 3. Light micrographs illustrating the inflammatory response in synovial membrane at 4 hours after carrageenan or vehicle injection. Four experimental situations are represented. It was observed that Carrageenan-evoked acute inflammatory response in which lining cell layer was composed of large cells that were irregularly layered and blended with subjacent infiltration with polymorphonuclear leukocytes. Pre-treatment of rats with iNOS inhibitor L-NIL (500 μg in 50 μl) reduced the number of polymorphonuclear cells in synovial membrane. (A) Saline alone group, (B) Saline + carrageenan group, (C) 7-Nitro + carrageenan group, (D) L-NIL + carrageenan group.

intra-articular pre-treatment of rats with a iNOS inhibitor L-NIL significantly prevented the reduction of weight load induced by carrageenan by 3 hours and 4 hours after carrageenan injection, however, a nNOS inhibitor 7-Nitro did not significantly prevent the reduction of weight load induced by carrageenan. Nevertheless, 7-Nitro had a tendency to block the reduction of weight load from 3~4 hours after carrageenan injection (Fig. 1).

Effects of intra-articular injection of different NOS inhibitors on inflammatory response

The injection of carrageenan increased the number of polymorphonuclear cells in synovial membrane. Fig. 2 shows the effects of different NOS inhibitors on inflammatory response. Pre-treatment of rats with L-NIL significantly reduced the number of polymorphonuclear cells in synovial membrane (70.75±2.44, n=5), compared to vehicle + carrageenan-treated group (87.67±4.51, n=5). However, pre-treatment of rats with L-NIL did not reduce the increased diameter of right knee joint induced by carrageenan injection (data not shown). Furthermore, 7-Nitro had no significant effects on the number of polymorphonuclear cells in synovial membrane (79.5±3.26, n=5).

In saline injected group, synovial membranes showed no inflammatory response in synoviocytes lying in a matrix which is exposed to the joint space. Lining cell layer was intact and almost devoid of an exudate or cellular infiltration (Fig. 3A). However, carrageenan-evoked acute inflammatory response was observed by 4 hours in which lining cell layer was composed of large cells that were irregularly layered and blended with subjacent infiltration with polymorphonuclear leukocytes (Fig. 3B). 7-Nitro, nNOS inhibitor had no significant effects on the number of polymorphonuclear cells in synovial membrane (Fig. 3C). Pretreatment with iNOS inhibitor (L-NIL) significantly reduced the number of polymorphonuclear cells in synovial membrane (Fig. 3D).

DISCUSSION

In this study, acute joint inflammation was induced by injecting carrageenan into the knee joint cavity of rats. Following induction of inflammation, the reduction of weight load was observed. The role of NO in modulating arthritic pain-related behavior and joint inflammatory response in acute arthritic animal was investigated by injecting two types of NOS inhibitors into the knee joint immediately prior to carrageenan injection.

These results demonstrate for the first time that intra-articular injection of L-NIL partially prevented the reduction of weight load on the inflamed side and the inflammatory response of synovial membrane, suggesting that L-NIL exhibits analgesic and anti-inflammatory properties when injected locally into the inflamed knee joints of rats. This finding provides evidence for the involvement of peripherally released NO in nociceptive transmission and inflammatory response in the acute inflammatory state. These findings are in agreement with the results by Honore and colleagues which showed that systemic administration of L-NAME reduced the paw edema induced by intraplantar injection of carrageenan (Honore et al., 1995, Chu et al., 2005).

It is likely that NO is released during the inflammation, inducing vasodilatation, and that it acts to increase vascular permeability to different chemical substances which activate specific receptors involved in pain signaling (Evans, 1995). The ability of iNOS inhibitor to cause vasoconstriction by inhibiting NOS may account for the peripheral anti-hyperalgesic and anti-inflammatory effects observed, since this may decrease the permeability of the blood vessel wall

to inflammatory mediators and algogenic substances, and prevent them from reaching their site of action. In the present study, pre-treatment of rats with L-NIL could not prevent the increase of knee joint diameter induced by carrageenan injection. This might have been low sensitivity of measurements with calipers. On the other hand, a direct action of NO on nociceptors cannot be ruled out. Recently, it has been shown that intracutaneous injection of NO evokes pain in humans in a dose dependent manner, suggesting that NO may activate cutaneous nociceptors directly or indirectly (Holthusen and Arndt, 1994). The pain response in these subjects, however, was not accompanied by two characteristics of the classical triple response: swelling or itching of the affected skin. Reddening of the skin, however, was observed around the NO injection site, indicating that flare is likely to be as a result of vasodilator action of NO rather than secondarily released mediators of inflammation. On the other hand, pain intensity in these subjects was shown to correlate with the locally applied dose of NO, suggesting that NO has a direct action on nociceptors rather than indirectly activating either a NO-triggered release of algogens or a blood flow-related alteration of their local concentrations (Holthusen and Arndt, 1994).

Although NO seems to play an important role as a peripheral mediator of pain and inflammation, the source of NO released during acute arthritis has not been elucidated. Since NO has a short half-life, it can not diffuse for a long distance. Thus, NO must necessarily be synthesized locally in the joint. Potential intra-articular sources of NO include the endothelial cells lining the synovial capillaries, neutrophils, infiltrating leukocytes and mesenchymal cells of the joint (Evans, 1995). Lymphocytes, mast cells, and macrophages produce considerable amounts of NO in inflamed joints (Stefanovic-Racic et al., 1994). Articular chondrocytes and synovial fibroblasts have also been shown to generate NO (Stadler et al., 1991).

Another possible source of NO in the joint would be the sensory endings of the primary afferent fibers. To determine whether the activation of nociceptors would release NO from the peripheral terminals of primary afferent fibers during inflammation, the selective NOS I inhibitor, 7-Nitro, was examined in this study. Previous studies have demonstrated that nNOS inhibitor exhibits antinociceptive activity, when i.p. injected into mice, by producing dose-related inhibition of the second phase of hindpaw licking following subplantar injection of formalin (Moore et al., 1993) and complete Freund adjuvant-induced persistent pain (Chu et al., 2005). However, the present results have shown that intra-articular injection of 7-Nitro had no effects on pain related behavior and was ineffective in preventing the inflammatory response usually seen as inflammation progresses.

Based on the assumption that nNOS is expressed in the peripheral terminals of primary afferent fibers, it is highly likely that there is very little activation of nNOS in the peripheral terminals of primary afferent fibers. It is also possible that NO generated from the nerves does not either diffuse enough to have local vascular effects or that the compound is rapidly metabolized. Nonetheless, in this study, L-NIL was shown to be very effective in preventing arthritic pain-related behavior as well as inflammatory response of synovial membrane.

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