Antihyperalgesic potentiating activity of L-NMMA via increasing IL-10 levels

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Abstract

Background and aim of the study: Previous studies showed that enhanced activity of inducible nitric oxide synthase (iNOS) has a deleterious effect on severity of arthritis in animal models. On the contrary, there is a conflicting notion that nitric oxide may be protective during inflammatory processes.The aim of the present study is to examine whether the suppression of NO production by L- NG - monomethyl arginine (L-NMMA), a non-selective NOS inhibitor can modify the potential anti-hyperalgesic effects of rolipram against mechanically-induced pain in rats.

Methods: Inflamed joints in rats were induced by intradermal injection of 0.1ml squalene before inoculation of complete Freund's adjuvant (CFA) into a different site in the subplantar surface of right hind paw. Pain threshold to pressure on hind paws was measured daily from day 0 until day 30 after adjuvant inoculation. Serum samples were taken for TNF-alpha and IL-10 assay.

Results: Simultaneous administration of 30 mg/kg/d L-NMMA with 3 mg/kg/d rolipram resulted in further significant reduction of hyperalgesia of left hind paw. Prophylactic rolipram in combination with 30mg/kg/d L-NMMA, significantly increase serum levels of interleukin- 10 (IL-10), however TNF-alpha levels are insignificantly changed.

Conclusion: L-NMMA, a NOS inhibitor potentiates anti-hyperalgesic and the immunomodulatory effect of rolipram, through increasing levels of the anti-inflammatory cytokine IL-10.

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Introduction

It has been suggested that endogenous production of nitric oxide (NO) is enhanced in proportion to the degree of inflammation in patients with rheumatoid arthritis (RA) owing to enhanced inducible nitric oxide synthase (iNOS) activity (Yki-Jarvinenet al.2003). Also, in osteoarthritis, NO production was found to be higher (Scher et al., 2007). It mediates many of the destructive effects of interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-alpha) in the cartilage and inhibitors of NO synthesis have demonstrated retardation of clinical and histological signs and symptoms in experimentally-induced osteoarthritis and other forms of arthritis (Vuolteenaho et al.,2007).

It appears likely that the increase in NO associated with arthritis can be caused by pro-inflammatory cytokines and mechanical stress and molecular oxygen is required for production of NO that is associated with osteoarthritis and RA (Fermor et al., 2007). Further evidence of the deleterious effects of NO comes from the study of Nagy et al. (2008) who supported the NO inhibiting therapeutic strategies for the treatment of chronic inflammatory diseases such as RA and concluded that local inhibition of NO synthesis at the site of synovial inflammation may provide better therapeutic tool than systemic inhibition. Their prior study revealed that overproduction of NO may perturb T cell activation, differentiation and effector response which may contribute in different ways to the pathogenesis of autoimmune diseases (Nagy et al., 2007).

Contrarily, there also exists the conflicting notion that NO may be protective during an inflammatory process. It has been shown that NO prevents apoptosis in rheumatoid synovial cells by directly inhibiting caspase-3-activation (Migita et al.,2001) and the local production of NO may be protective by the virtue of its ability to regulate the release of pro-inflammatory mediators (Paul-Clark ,2001). In addition, NO donors were found to increase the production of hyaluronic acid by synovial cells from patients with RA (Chenevier-Gobeaux et al., 2004). Other studies found that NO does not mediate the chronic inflammation and joint destruction which occur during the latter phase and the therapeutic administration of a selective inhibitor of iNOS does not ameliorate the chronic inflammation and tissue damage associated with adjuvant arthritis in rats (Fletcher et al., 1998). Furthermore, it has been shown that NO has limited modulating effects in cartilage metabolism with evidence for both protective and deleterious effects (Jang and Murrell, 1998) and no fundamental relationship between magnitude of NO production and arthritis susceptibility and severity suggesting that NO has no effector role in arthritis (Miletie et al., 2007). Similarly, it has been found that the relationships between measures of arthritis disease activity and urinary and serum nitric oxide levels were not significant in rheumatoid patients (Weinberg et al., 2007).

Interesting studies have implicated that NO has dual effects. It has been reported that relatively low concentration of NO plays a defensive role in the immune system (Hobbs et al.,1999) and exerts anti-apoptotic effects via cGMP (Dimmeler et al.,1997) while higher concentration causes numerous pathological processes including inflammation (Bogdan,2001), vascular damage (Luoma et al., 1998) and apoptosis in various cell types (Arstall et al.,1999). Additionally, low concentration of SNP suppresses subsequent high concentration SNP-induced apoptosis by inhibiting p38 kinase (Kwak et al., 2006).

PDE inhibitors as a possible agent against the other chronic inflammatory diseases such as RA, because of the elevation of intracellular level of cyclic AMP in leukocytes which is accompanied by inhibition of production of TNF-alpha (Teixeira et al., 1997). It has been reported that both nonselective as well as PDE4 specific inhibitors were effective in ameliorating autoimmune disease in different experimental autoimmune encephalomyelitis models (Sommer et al., 1995) and collagen-induced arthritis models (Nyman et al., 1997). However, the therapeutic utility of PDE4 inhibitors and their new structural classes to suppress inflammation has not been disclosed till now due to lack of tolerability (Span, 2008; Giembycz, 2008).

PDE4 inhibitors reduce the synthesis and release of proinflammatory mediators, cytokines and active oxygen species .These effects on immunocompetent cells may explain the anti-inflammatory and bronchodilatatory effects induced by PDE4 inhibitors in animal models of inflammatory diseases (Souness et al., 2000 and Huang et al., 2001).

In the present study, we examined whether the suppression of NO production by L- NG - monomethyl arginine (L-NMMA), a non-selective NOS inhibitor can modify the potential anti-inflammatory effects of rolipram in adjuvant arthritic rats.

Materials and Methods

Animals

The experimental study was carried out using adult female albino rats of the Sprague-Dawely strain weighing between 160-200 grams. The animals were acclimatized in a light- and temperature- controlled room (23±1°C) with a 12-12 hr dark-light cycle. The rats were fed with commercial pelleted rat feed and water was given *ad libitum*. Food was placed on the floor of the cage to facilitate access, as the pain which accompanies adjuvant-induced arthritis renders the rats immobile and unable to use their hind limbs to obtain food from the cover mesh of the cage. The experimental protocol was approved by the local ethical committee.

Reagents

Complete Freund's adjuvant (CFA) was purchased from Difco laboratories, Detroit, Michigan, USA. Squalene was purchased from MP Biomedicals, Inc. Rolipram and NG-monomethyl-L- arginine (L-NMMA) were purchased from Sigma chemical, St.Louis, USA. L-NMMA was freely dissolved in water. Rolipram was dissolved in 1% diluted DMSO.

Experimental induction of polyarthritis

Rat model of AIA, induced by the administration of Freund's adjuvant, has been used extensively in studying the roles of autoimmunity and inflammation in the pathogenesis of joint disease. It exhibits several pathological changes similar to those occurring in RA (Weichmann, 1989). Preliminary experiments showed that signs of arthritis did not appear in the contralateral non-injected hind paws after CFA inoculation. Also, the use of squalene, a known adjuvant for induction of arthritis (Carlson et al., 2000), alone failed to induce arthritis in the contralateral hind paw. So, the method of induction of adjuvant arthritis by Trentham et al (1977) was modified by intradermal injection of 0.1ml squalene before inoculation of CFA

into a different site in the subplantar surface of right hind paw to increase the sensitivity of rats to CFA. Squalene was also used by others to potentiate the effect of CFA (Santos and Tipping, 1994). Each animal in all groups was injected with 0.1ml squalene and 0.1 ml CFA except animals of control non-adjuvant group. The day of inoculation was regarded as day 0 while day 16 was the day in which edema in the contralateral, non-injected, hind paw was observed.

Pain threshold to pressure on hind paws was measured daily from day 0 until day 30 after adjuvant inoculation. At the end of the study, the animals were sacrificed and the blood was collected. Blood samples were immediately centrifuged at 3000 rpm for 10 minutes and serum samples were stored at -80°C until assayed for TNF-alpha and IL-10.

Design of experimental groups

Two groups (I&II) of 6 animals each served as control non-adjuvant and adjuvant non-treated arthritic rats received saline intraperitoneally (i.p.) daily. Other animals were randomly allocated into two treatment protocols (prophylactic or therapeutic). Each treatment protocol contains 6 groups of 6 animals each. Drug treatment was started on day 5 till day 14 in prophylactic protocol and on day 16 till day 25 in therapeutic protocol. Groups IV, V and VI in each protocol received i.p. rolipram alone in doses of 4.5,3 and 1.5 mg/kg/d respectively. Groups VII were treated i.p. with 3 mg/kg/d rolipram combined with 30 mg/kg/d L-NMMA. Rats of groups III were given orally 1 ml of DMSO (1% diluted in water).

Experimental measures

For each rat in the previously described experimental groups, the following evaluation parameters were daily measured till day 30 after disease induction:

Analgesimetry

Using a Ugobasile analgesimeter (UgoBasile Biological Research Apparatus, Italy), a crescent pressure (in grams) was applied separately to the posterior paws until the animal displayed a reaction that consisted of withdrawing the paw and/or vocalizing (Andersen and Tufik, 2000). The slide of the device moved at the speed of 16mm per second. The force on the paw was at rate of 16 grams per second, so a distance of 11.5mm means 115 grams. The pain threshold to pressure (gm) on hind paws of rats was measured. The following formula was used to calculate the percentage of change of pressure (gm) on hind paws on day 30 for each animal:

(Pressure on day 30 – pressure before adjuvant injection on day 0) X 100/ Pressure before adjuvant injection on day 0

Measurement of cytokines

Animals were sacrificed on day 30 after disease induction and samples of blood were taken to separate sera from control ,adjuvant non-treated and SNP-treated arthritic rats .Serum levels of TNF- alpha and IL-10 were determined using enzyme-linked immunosorbent assay (ELISA) kits from (Bender Medsystems,Vienna, Austria). Antibodies specific for rat TNF-alpha and IL-10 were coated onto the wells of the microtiter strips and the samples including standards of known rat TNF-alpha and IL-10 were pipetted into the wells, incubated and washed. Intensity of the color was determined at (450) nm with a correction wave length of (630) nm.

Statistical Analysis

The results are presented as the mean ±standard error. Percentage of change of pressure (gm) on hind paws and serum levels of cytokines, measured in different treatment groups, were compared with control groups by one way analysis of variance (ANOVA) and Student's t-tests for significance. Also, significance tests were calculated to determine the differences between the effects of different doses of rolipram alone against its combination with L-NMMA.

Results

Effect of pretreatment of rats with L-NMMA on pain threshold of arthritic rats

Adjuvant inoculation into control rats (group II) was accompanied by hyperalgesia as evidenced by lowering of the pain threshold to pressure (gm) on hind paws. The animals presented a reduction of pain threshold until the end of experiments on day 30. On day 30 after adjuvant inoculation, the percentage of reduction of pressure (gm) on right and left hind paws were 54.9 ±0.3 and 62±0.4 respectively as compared with pressure at day 0 before adjuvant injection. Prophylactic and therapeutic administration of rolipram alone in groups IV, V and VI markedly ($P<0.05$) decreased hyperalgesia of the arthritic rats by increasing the pain threshold to pressure on both hind paws (by decreasing the percentage of reduction of pressure). On day 30, the percentages of decrease of pressure on right hind paws of these groups were 3.5±0.1, 4.2±0.1 and 6.2±0.1 in prophylactic protocol and 4.1±0.02, 7±0.1 and 8.2±0.01 in therapeutic protocol respectively. The inhibitory effect of prophylactic and therapeutic 3 mg/kg/d rolipram on hyperalgesia of arthritic rats was significantly ($P<0.05$) enhanced by simultaneous administration of 30mg/kg/d L-NMMA (1.2±0.01 and1.7±0.01 respectively) (Table1A).

Therapeutic and prophylactic administration of rolipram was also effective in reducing the hyperalgesia of left hind paw induced by adjuvant inoculation.

Percentages of decrease of pressure were 5.2±0.01, 6.1±0.04, 8.2±0.02 in groups IV, V and VI respectively. Simultaneous administration of 30 mg/kg/d L-NMMA with 3 mg/kg/d rolipram resulted in further significant reduction of hyperalgesia of left hind paw.

Prophylactic and therapeutic DMSO protocols reduced hyperalgesia of right and left hind paws of arthritic rats compared with control adjuvant arthritic group. However, rolipram in higher doses (4.5 and 3 mg/kg/d) were significantly more effective in reducing hyperalgesia compared with DMSO (Table 1B).

Effect of pretreatment of rats with L-NMMA on serum TNF-alpha and IL-10

As shown in Table B , serum TNF-alpha level was insignificantly and slightly greater in adjuvant arthritic non-treated control rats (group II) than that of control non-adjuvant saline-treated animals on day 30 after adjuvant inoculation (31.04±1.4 picograms, $P>0.05$ in group II compared with 30.7 ±2.3 picograms in saline-treated rats, group I). Serum IL-10 level was significantly lower ($P<0.05$) in adjuvant non-treated control rats, 171.6±34 picograms, $P<0.05$ compared with 345.6±64.4 in saline- treated rats).

TNF-alpha levels in sera , obtained on day 30 after adjuvant inoculation, of arthritic rats treated with 3 mg/kg/d rolipram alone, either prophylactically or therapeutically (groups V) , or prophylactically in combination with 30 mg/kg/d L-NMMA (group VII),were insignificantly changed ($P>0.05$) compared with either saline-treated non-adjuvant (group I) or adjuvant arthritic non-treated control (group II). They were 27.5±1.5, 30.1±1.8, 29.6±2.8 and 29.04±1.2 picograms respectively.

Serum levels of IL-10 were insignificantly higher ($P>0.05$) in rats given prophylactic rolipram alone (382 ±97.04) while they were significantly higher ($P<0.05$) in rat groups given either therapeutic rolipram alone (454.2±150.2) or prophylactic rolipram in combination with 30mg/kg/d L-NMMA (529±129.7) compared with those in adjuvant arthritic control rats (group II) . However, IL-10 levels in the aforementioned groups were insignificantly higher ($P>0.05$) compared those of saline-treated non adjuvant arthritic animals (group I).

Table 1 A: Effect of prophylactic administration of rolipram on the % of change to pressure (gm) on the right hind paw of adjuvant arthritic rats

Drug treatment	% of change to pressure (gm) on the right hind paw			
	Day 5	Day 9	Day 14	Day 30
Saline-treated non adjuvant rats (group I)	4.6±0.01	0	3.5±0.1	2.3±0.1
Adjuvant non- treated arthritic rats (group II)	39.4±0.2	33.8±0.3	26.7±0.4	54.9±0.3
Vehicle-treated (1% DMSO) adjuvant arthritic rats (group III)	42.5±0.1	29.8±0.1	18.6±0.1	7.5±0.1*
Rolipram-treated (4.5mg/kg/d) arthritic rats (group IV)	38.5±0.1	13.5±0.1	11.9±0.1* º	3.5±0.1* º
Rolipram-treated (3mg/kg/d) arthritic rats (group V)	37±0.3	22.2±0.1	12.3±0.2* º	4.2±0.1* º
Rolipram-treated (1.5mg/kg/d) arthritic rats (group VI)	41.5±0.1	28.3±0.2	17.2±0.1*	6.2±0.1*
Rolipram (3mg/kg/d) in combination with L-NMMA (30mg/kg/d) (group VII)	**46±0.3**	**27±0.1**	**5.5±0.3* º†**	**1.2±0.01* º†**

Values represent the mean±SE.* p<0.05 vs. groups II, º p<0.05 vs. groups III, † p<0.05 vs. groups V, ANOVA.

Table 1 B: Effect of therapeutic administration of rolipram on the % of change to pressure (gm) on the right hind paw of adjuvant arthritic rats

Drug treatment	% of change to pressure (gm) on the right hind paw			
	Day 15	Day 20	Day 25	Day 30
Saline-treated non adjuvant rats (group I)	3.5±0.1	4.6±0.1	8±0.1	2.3±0.1
Adjuvant non- treated arthritic rats (group II)	29±0.4	44±0.2	46±0.2	54.9±0.3
Vehicle-treated (1% DMSO) adjuvant arthritic rats (group III)	8±0.1	4±0.1	19.2±0.1	13.1±0.1*
Rolipram-treated (4.5mg/kg/d) arthritic rats (group IV)	24±0.1	14.5±0.2	11.1±0.1* º	4.1±0.02* º
Rolipram-treated (3mg/kg/d) arthritic rats (group V)	19±0.1	15±0.1	13±0.1* º	7±0.1* º
Rolipram-treated (1.5mg/kg/d) arthritic rats (group VI)	20±0.1	16±0.1	14.1±0.1* º	8.2±0.01* º
Rolipram (3mg/kg/d) in combination with L-NMMA (30mg/kg/d) (group VII)	**37±0.3**	**27±0.2**	**2±0.01* º†**	**1.7±0.01* º†**

Values represent the mean±SE.* p<0.05 vs. groups II, º p<0.05 vs. groups III, † p<0.05 vs. groups V, ANOVA.

Table 2: Effect of rolipram either alone or in combination with L-NMMA on serum levels of TNF-α and IL-10 in adjuvant arthritic rats

Group	Drug treatment	Serum levels (picograms)	
		TNF- α	IL-10
I	Saline – treated (non- adjuvant)	30.7±2.3	345.6±64.4
II	Adjuvant arthritic (non-treated)	31.04±1.4	171.6±34†
V	Rolipram-treated [3mg/kg/d] (Prophylactic protocol)	27.5±1.5	382±97.04
V	Rolipram-treated [3mg/kg/d] (Therapeutic protocol)	30.1±1.8	454.2±150.2*
VII	Rolipram [3 mg/kg/d] in combination with L-NMMA [30 mg/kg/d](Prophylactic Protocol).	29.04±1.2	529±129.7*

Samples were taken from rats on day 30 after adjuvant inoculation. Values represent the mean±SE. * P <0.05 for groups V and VII vs. group II, † P <0.05 for group II vs. group I, ANOVA.

Discussion

Our study demonstrated a highly significant potentiating effect for NG- monomethyl-L-arginine, a non-selective NOS inhibitor, to the inhibitory effect of rolipram on progression of adjuvant arthritis in rats. Previous studies reported that increased NO production and iNOS activation contributed to the high levels of apoptosis of synovial lining cells and chondrocytes in tissues samples from patients with rheumatoid arthritis (Firestein et al.,1995). Other ex vivo studies showed that the NOS inhibitor L-NMMA exerted dramatic inhibitory effects on apoptosis in explants of both synovium and cartilage, which were reversed by the NO donor S-nitrosoacetylpenicillamine (SNAP) (Van't Hof et al., 2000). Other workers have shown that NO induces apoptosis in cultured chondrocytes (Blanco et al., 1995). Our observation is consistent with McCartney-Francis et al. (1993) that demonstrated an

inhibitory effect of L-NMMA on the tissue damage associated with streptococcal cell wall-induced arthritis when administered either prophylactically or therapeutically.

Inhibition of leukotriene B4 (LTB4) (Griswold et al.,1993), interferon-gamma (IFNγ) (Essayan et al., 1997), tumor necrosis factor-alpha (TNF- α) (Singh et al.,1997), interleukin-4 (IL-4) and interleukin-5 (IL-5) (Essayan et al.,1997), increase of interleukin-10 (IL-10) release and suppression of T-lymphocyte function, as well as direct, protective effects on cartilage and bone (Souness and Foster,1998), are all potential mechanisms by which the anti-inflammatory effects of roliparm are mediated. Inhibition of (TNF- α) is especially important because of the pivotal role this cytokine plays in inflammatory processes by attracting leukocytes (Pettipher et al., 1996), activating endothelial cells (Shimmer et al., 1995) and contributing to edema (Sekut et al., 1995).

The present study demonstrated that prophylactic or therapeutic administration of rolipram did not alter significantly the serum level of TNF-α. Regarding IL-10, our study demonstrated a significantly augmenting effect of treating adjuvant arthritic rats with either rolipram alone, from day 16 to day 25 after disease induction in a dose of 3 mg/kg/d given orally, or in combination with non-selective NOS inhibitor, L-NMMA (30 mg/kg/d given intraperitoneally) from day 5 to day 14 after adjuvant inoculation. Serum levels of IL-10 in the aforementioned groups were significantly higher compared to levels in adjuvant non-treated animals. This confirmed the anti-inflammatory activity of IL-10 in adjuvant- induced arthritis in rats and in agreement with other previous studies. Hisadome et al., (2000) reported that a novel antirheumatic drug, Y-39041, has an anti-arthritic effect through not only TNF- α and interlukin-6 suppression but also interleukin-10 augmentation. Eigler et al., (1998) demonstrated that cAMP- elevating agents like rolipram enhance lipopolysaccharide- induced IL-10 synthesis and suppress TNF- α production. Also, Kambayashi et al., (2001) reported that cAMP-elevating agents, especially PDE inhibitors, increase IL-10 and inhibit TNF- α and IL-12 production and these drugs shift the immune response towards a Th2 phenotype. Autoimmune disease models which are Th1- mediated such as collagen-induced arthritis (Nyman et al., 1997) have been successfully treated with PDE 3 and 4 inhibitors. On the contrary, Jimenez et al., (2001) have found that the specific inhibition of PDE4 by rolipram reduces the production of several cytokines such as IL-5, IL-10, TNF- α and IL-2 but poorly affects IFN-γ and T-cell proliferation in response to activation by anti- CD3. Other reports indicated that PDE inhibitors, such as pentoxifylline and rolipram, also inhibited IL-4, IL-5 and IL-10 secretion by T cells (Foissier et al., 1996).

In conclusion, our results presented in this study revealed that systemic use of rolipram either alone or in combination with the NOS inhibitor, L-NMMA, significantly ameliorated adjuvant-induced arthritis in rats.

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