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Diacerein suppresses the increase in plasma nitric oxide in rat adjuvant-induced arthritis

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Abstract

We investigated the effects of rhein, an active metabolite of diacerein, on the interleukin-1α-stimulated production of nitric oxide (NO) in rabbit articular chondrocytes, and the effects of diacerein on NO production in rat adjuvant-induced arthritis. At doses of 10 and 30 μM, rhein significantly inhibited the interleukin-1α-stimulated NO production in chondrocytes. In the rat adjuvant-induced arthritis model, diacerein was administered for 21 days, starting at the time of adjuvant injection. Paw swelling and plasma NO level were measured in order to assess the effect of diacerein on arthritis and NO biosynthesis in the whole body. At doses of 30 and 100 mg/kg/day, diacerein significantly suppressed the development of adjuvant-induced arthritis and the increase in plasma NO. These results suggest that the inhibitory effect of diacerein on rat adjuvant-induced arthritis is partly related to its reduction of the NO production induced by adjuvant-induced arthritis.

Keywords: Diacerein; Rhein; Nitric oxide (NO); Chondrocyte; Adjuvant-induced arthritis

1. Introduction

Inflammatory mediators such as interleukin-1 are thought to play major roles in joint diseases such as osteoarthritis and rheumatoid arthritis (Kirkham, 1991). Interleukin-1 has been shown to inhibit extracellular matrix synthesis, induce matrix metalloproteinases (Arend and Dayer, 1990), and stimulate the degradation of proteoglycans (Krakauer et al., 1985; Tyler, 1985), resulting in damage to both cartilage and bone. One of the mechanisms by which interleukin-1 elicits its proinflammatory effects is by stimulating the production of nitric oxide (NO). Inducible NO synthases (iNOS) are only found in tissues after its induction, such as in response to proinflammatory cytokines (Stadler et al., 1991). NO produced in response to cytokine stimulation exerts a number of catabolic effects that are expected to promote the degradation of articular cartilage: inhibition of collagen and proteoglycan synthesis (Taskiran et al., 1994; Stefanovic-Racic et al., 1996, 1997), and induction of apoptosis (Lotz et al., 1999). Indeed, the level of NO in the serum and synovial fluid of patients with osteoarthritis or rheumatoid arthritis has been shown to be significantly increased (Kaur and Halliwell, 1994). Inducible NOS inhibitors have been found to significantly reduce the NO production associated with rat adjuvant-induced arthritis (Ialenti et al., 1993; Connor et al., 1995) and the progression of experimental osteoarthritis in dogs (Pelletier et al., 1998a).

Clinical studies have suggested that diacerein exerts a beneficial effect on the symptoms of osteoarthritis (Nguyen et al., 1994; Pelletier et al., 2000). Diacerein shows anti-arthritic and chondroprotective effects in an animal osteoarthritis model (Brandt et al., 1997). Diacerein also prevents cartilage breakdown by reducing the levels of proinflammatory cytokines (Moore et al., 1998). Diacerein is known to be completely metabolized by animals and humans into rhein, an active metabolite of diacerein (Debord et al., 1994). Rhein can down-regulate the interleukin-1α-induced production of pro-matrix metalloproteinases by rabbit articular chondrocytes (Tamura and Ohmori, 2001). However, the exact mechanisms of the therapeutic action of diacerein on osteoarthritis are not clear.

We investigated the effects of rhein, an active metabolite of diacerein, on the recombinant human interleukin-1α-stimulated production of NO in cultured rabbit articular chondrocytes, and the in vivo effects of diacerein on NO production in rat adjuvant-induced arthritis.

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2. Materials and methods

2.1. Materials

Diacerein was provided by Proter (Milan, Italy). Rhein sodium salt and L-N^6-(1-iminoethyl)lysine hydrochloride were synthesized in our laboratories. Naproxen was purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and fungizone® were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum was purchased from Biocell Laboratories (Rancho Dominguez, CA, USA). Recombinant human interleukin-1α was purchased from Genzyme (Cambridge, MA, USA). Actinase E was purchased from Kaken Pharmaceuticals (Tokyo, Japan). Collagenase P and nitrate reductase were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Sodium nitrite, flavin adenine dinucleotide (FAD) and pyrvate were purchased from Wako (Osaka, Japan). Mycobacterium butylicum was purchased from Difco Laboratories (Detroit, MI, USA). Nicotinamide adenine dinucleotide phosphate reduced form and lactic dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan).

2.2. Animals

Female Japanese White rabbits (3-week-old) were purchased from Ichikawaya (Tokyo, Japan) and female Lewis rats (7-week-old) were purchased from Charles River Japan (Kanagawa, Japan). They kept at the specific pathogen-free animal facility where a temperature of 22–24°C, a humidity of 50–60%, and a 12-h day/night cycle were maintained constantly.

2.3. Culture of rabbit articular chondrocytes

Articular chondrocytes were isolated from the shoulder and knee joints of rabbits. Cartilage was digested with 0.4% actinase E for 1 h at 37°C followed by 0.025% collagenase P for 5 h at 37°C. The viability of the harvested cells as assessed by Trypan blue exclusion was always $>95\%$. The chondrocytes were then suspended in DMEM with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone®). The chondrocytes were cultured in each well of 24-well tissue culture plates at a density of 1×10^5 cells/ml under 5% CO_2–95% air at 37°C. Primary cultures maintained in a monolayer were used throughout the study. After cells reached confluence, the medium was changed to DMEM with 2% fetal bovine serum and the antibiotics. Confluent chondrocytes were then incubated with rhein sodium salt, naproxen or L-N^6-(1-iminoethyl)lysine hydrochloride in the presence of 0.5 ng/ml of interleukin-1α for up to 4 days, and the medium and additives were then changed every 2 days. After the incubation period, media were collected and stored frozen at −40°C. Rhein sodium salt or L-N^6-(1-iminoethyl)lysine hydrochloride was dissolved in distilled water, and naproxen was dissolved in dimethyl sulfoxide, then they were added to the cell cultures at appropriate concentrations. The final concentrations of distilled water and dimethyl sulfoxide were 0.1% and 0.01%, respectively. The concentrations of rhein used were chosen to reflect levels that are below, at, or above the established therapeutic anti-inflammatory serum values described in clinical practice (Debord et al., 1994). Rhein, naproxen or L-N^6-(1-iminoethyl)lysine hydrochloride had no effect on chondrocyte viability in this study.

2.4. Induction of adjuvant-induced arthritis in rats

Arthritis was induced on day 0 by the injection of 0.1 ml of adjuvant, a 0.6% suspension of heat-killed mycobacterium butylicum in liquid paraffin, into the footpad of the right hind paw of rats under light ether anesthesia. Foot volumes were measured using a plethysmometer, TK-101 (Unicom, Chiba, Japan), before and after adjuvant injection. Diacerein and naproxen were suspended in 5% arabic gum solution for oral administration. L-N^6-(1-iminoethyl)lysine hydrochloride was dissolved in saline for subcutaneous administration. Drugs were administered once a day for 21 successive days, starting immediately after the injection of adjuvant. A 50-μl of blood was collected under deep anesthesia by orbital bleeding into microfuge tubes containing heparin sodium. Plasma was prepared by centrifugation and was stored at −80°C until use.

2.5. Determination of no production

The concentration of NO in the conditioned medium was determined based on the Griess reaction with sodium nitrite as standard (Green et al., 1982). Briefly, the culture samples or sodium nitrite standard dilutions were mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2% H_3PO_4) and incubated for 10 min at room temperature. The optimal density (OD) was measured at 540 nm using a microplate spectrophotometer, THERMOmax™ (Molecular Devices, Sunnyvale, CA, USA). The concentration of NO was calculated by using a standard curve of sodium nitrite ranging from 0.625 to 40 μM.

The concentration of NO in the plasma was measured as described earlier (Tracey et al., 1995). Briefly, nitrates in the plasma were enzymatically reduced to nitrite with nitrate reductase. A 20-μl of plasma was mixed with 10 μl each of 0.1 mM FAD, 1 U/ml nitrate reductase and 2.5 mM nicotinamide adenine dinucleotide phosphate reduced form in 100 mM phosphate buffer (pH 7.4) in individual wells of a 96-well plate. The reaction was allowed to proceed for 3 h in the dark. To each sample, 50 μl of 40 U/ml lactic dehydrogenase in 50 mM pyrvate was added. A 100-μl of Griess reagent was added to each well, and the OD_{540} was measured using a microplate spectrophotometer. Nitrite concentrations were calculated by us-

ing a standard curve of sodium nitrite ranging from 1 to 100 μM.

2.6. Statistical analysis

The data were analyzed by the *F*-test followed by the Aspin–Welch test. Multiple comparisons between treatment groups were assessed by a one-way analysis of variance test followed by the Dunnett test. Dose dependence was assessed by max *t*-test followed by Williams test. A P value of less than 0.05 was considered to be statistically significant. The data are presented as the means ± S.D.

3. Results

3.1. Effects of rhein on the interleukin-1α-stimulated NO production by chondrocytes

Initially, chondrocytes were treated with various concentrations of interleukin-1α (data not shown). Chondrocytes spontaneously produced only low amounts of NO. The concentration of interleukin-1α (0.5 ng/ml) used in this study caused submaximal effects on the production of NO. The addition of interleukin-1α provoked a large increase in NO production (Table 1). L-N^6-(1-iminoethyl)lysine hydrochloride, the iNOS inhibitor, significantly suppressed interleukin-1α-stimulated NO production at 3 and 10 μM ($P = 0.0027$ and $P < 0.0001$). Rhein was active at doses between 1 and 30 μM and showed significant inhibition at 10 and 30 μM ($P < 0.0001$ and $P < 0.0001$) whereas naproxen (0.1 to 10 μM) had no effect.

Table 1
Effects of rhein, naproxen and L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL) on interleukin-1α-stimulated NO production in cultured rabbit articular chondrocytes
Confluent chondrocytes were treated with drugs in the presence of interleukin-1α (IL-1α, 0.5 ng/ml) for 4 days. The concentration of NO released into the culture medium was determined using the Griess reaction as described in Materials and methods. Each value represents the mean ± S.D. for four wells.

Treatment	Concentration (μM)	NO (μM)
None	–	0.00 ± 0.00
IL-1α	–	20.92 ± 1.89
+ Rhein	1	19.78 ± 1.32
+ Rhein	3	19.80 ± 2.05
+ Rhein	10	10.25 ± 1.00[a]
+ Rhein	30	5.31 ± 0.24[a]
IL-1α	–	21.18 ± 2.11
+ Naproxen	0.1	21.49 ± 2.02
+ Naproxen	1	21.12 ± 1.56
+ Naproxen	10	22.88 ± 1.12
IL-1α	–	21.72 ± 1.83
+ L-NIL	1	20.87 ± 1.32
+ L-NIL	3	18.48 ± 0.52[b]
+ L-NIL	10	13.87 ± 1.31[a]

[a] $P < 0.001$ significantly different from IL-1α control (Williams test).
[b] $P < 0.01$ significantly different from IL-1α control (Williams test).

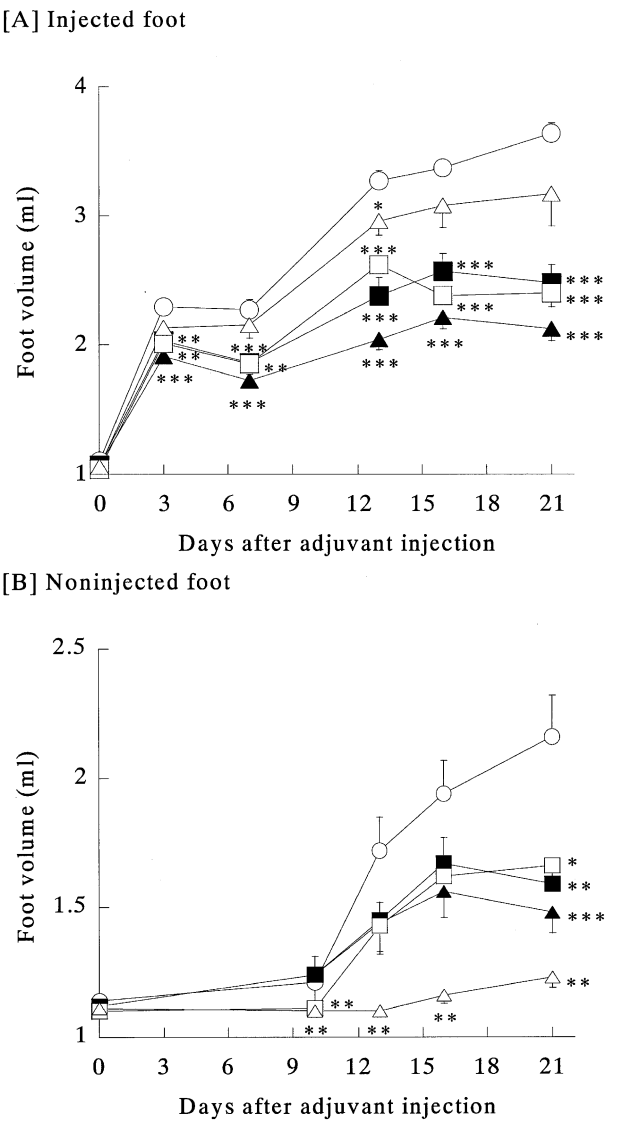

Fig. 1. Effects of diacerein, naproxen and L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL) on paw swelling in adjuvant-induced arthritis rats. On day 0, adjuvant was injected into the footpad. At intervals thereafter, the volume of the injected (A) or noninjected (B) paw was measured using a plethysmometer. Drugs were administered once a day for 21 successive days, starting immediately after adjuvant injection. ○; control, ■; 30 mg/kg/day of diacerein, ▲; 100 mg/kg/day of diacerein, □; 3 mg/kg/day of naproxen, △; 10 mg/kg/day of L-NIL. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ significantly different from control. Each value represents the mean ± S.D. from six rats per group.

3.2. Effects of diacerein on rat adjuvant-induced arthritis

The effect of diacerein on the development of rat adjuvant-induced arthritis was investigated. Paw swelling and plasma NO levels were measured to assess the effect of diacerein on the arthritic response and whole-body NO production, respectively. Swelling was evident within 1

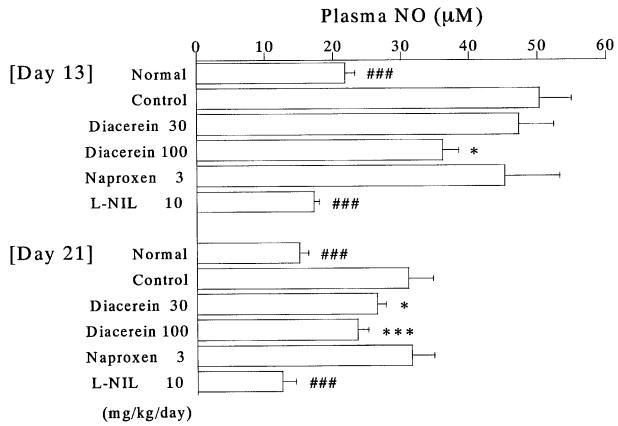

Fig. 2. Effects of diacerein, naproxen and L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL) on the plasma levels of NO in adjuvant-induced arthritis rats. On day 0, adjuvant was injected into the footpad of the right hind paw. On days 13 and 21, the concentration of NO in the plasma was measured by the Griess reaction as described in Materials and methods. Drugs were administered once a day for 21 successive days, starting immediately after adjuvant injection. $^{*}P < 0.05$; $^{***}P < 0.001$ significantly different from control. $^{###}P < 0.001$ significantly different from normal. Each value represents the mean ± S.D. from six rats per group.

day in the injected paw and peaked on day 3 and began to decline through day 7. The chronic phase of the inflammatory response began to occur 7 days after adjuvant injection (Fig. 1). The concentration of NO in the plasma was significantly higher on days 13 and 21 than in the normal rats ($P = 0.0007$ on day 13 and $P = 0.0001$ on day 21). The maximal plasma NO levels were elevated 2.3 times the normal level on day 13 (Fig. 2).

The administration of diacerein (30 and 100 mg/kg/day) on days 0 to 20 suppressed the volume of both the primary and the secondary swelling of the adjuvant-injected and noninjected paws (Fig. 1A, B). Diacerein caused a dose-related reduction in the plasma NO elevations compared with the vehicle (on day 13, $P = 0.0352$ at 100 mg/kg/day, and on day 21, $P = 0.0193$ at 30 mg/kg/day and $P = 0.0004$ at 100 mg/kg/day) (Fig. 2). The administration of L-N^6-(1-iminoethyl)lysine hydrochloride (10 mg/kg/day) slightly reduced the swelling of the injected paw, but significantly on day 13 ($P = 0.0486$) (Fig. 1A). However, it caused a significant inhibition of the swelling of the noninjected paw (Fig. 1B). In addition, the L-N^6-(1-iminoethyl)lysine hydrochloride treatment reduced the plasma NO level to the level of the naive controls ($P = 0.0005$ on day 13 and $P < 0.0001$ on day 21) (Fig. 2). The administration of naproxen (3 mg/kg/day) significantly inhibited the swelling of the paws whereas it had no effect on the plasma NO elevation (Figs. 1 and 2).

4. Discussion

The overproduction of NO seems to be an important factor in the pathology of osteoarthritis. Compared to normal samples, the level and expression of iNOS is higher in the osteoarthritis synovium (McInnes et al., 1996) and cartilage (Amin et al., 1995). Moreover, increased NO levels have been found in the plasma and urine of rats during adjuvant-induced arthritis (Cannon et al., 1996). The involvement of NO in the pathophysiology of osteoarthritis might present a potential new target for pharmacological intervention in this disease.

In the present study, we chose rabbit articular chondrocytes because they are among the main producers of NO within the osteoarthritis-affected joint. NO is produced by articular chondrocytes in large amounts by iNOS in response to activation by interleukin-1 and other agents. As observed in the experiments measuring interleukin-1α-stimulated NO production, rhein inhibited NO production. This effect of rhein was dose dependent. Interleukin-1α-stimulated NO production was reduced significantly by drug concentrations well within the therapeutic range in osteoarthritis patients treated with diacerein. NO production may have been inhibited by a variety of mechanisms including regulation of the production or activity of iNOS. The effect of rhein appeared to result from iNOS transcriptional and/or post-transcriptional events, as indicated by a decrease in the levels of mRNA and protein for this enzyme in human osteoarthritis chondrocytes (Pelletier et al., 1998b). The non-steroidal anti-inflammatory drug, naproxen, had no effect on NO production. This finding clearly indicates that these two classes of drugs used in the treatment of osteoarthritis have different mechanisms of action.

We investigated the in vivo effects of diacerein on NO production in rats with adjuvant-induced arthritis. Adjuvant-induced arthritis in the rat is a model of chronic inflammation that exhibits several pathological changes similar to those occurring in rheumatoid arthritis in humans, which is characterized by chronic inflammation of the joints. Previous reports have implied that iNOS has a role in the development of inflammation based on the effects of iNOS inhibitors (Ialenti et al., 1993; Stefanovic-Racic et al., 1995; Connor et al., 1995). A decrease in the plasma or urinary NO levels after administration of these inhibitors correlated with a decrease in inflammatory parameters (Stefanovic-Racic et al., 1995). The administration of diacerein was efficacious in preventing the swelling of the adjuvant-injected and noninjected paws. Moreover, we demonstrated, for the first time, that diacerein suppressed the increase in plasma NO level during the development of adjuvant-induced arthritis. The iNOS of neutrophils, chondrocytes and synoviocytes is known to be up regulated during inflammation (Stefanovic-Racic et al., 1993). Thus, the relative increase in plasma NO during adjuvant-induced arthritis may be indicative of the activity of these cells, as well as the joint damage that appears during the disease. Studies have shown that diacerein and rhein do not reduce prostaglandin E_2 synthesis, and may even have the opposite effect of increasing it (Franchi-

Micheli et al., 1983; Pelletier et al., 1998b). The inhibition of NO production in vitro also occurred in vivo, and thus might present a new mechanism that could in part explain the improvement seen in joint diseases treated with diacerein.

The increases in plasma NO concentrations found in rats with adjuvant-induced arthritis are consistent with previously reported data for patients with arthritis. These patients were found to have elevated serum as well as synovial fluid levels of NO compared to normal controls (Farrell et al., 1992). The changes in plasma NO levels occurred earlier than paw swelling in rats with adjuvant-induced arthritis (Connor et al., 1995). The swelling of the paw was induced or continued even though plasma NO levels had not reached a maximum. These results clearly indicate that the timing of NO involvement in the development of adjuvant-induced arthritis is different from that of paw swelling. NO was shown to be involved in the development of adjuvant-induced arthritis, possibly by interfering with the activation of T lymphocyte and/or macrophages (Ialenti et al., 1993). Thus, NO inhibition may affect the function of T cells during the elicitation of the immune response necessary for the development of chronic inflammation and arthritis. However, the action of diacerein on the function of T cells is not clear. Further studies of whether the inhibitory action of diacerein is associated with its immunoregulatory action may be necessary.

In conclusion, we have demonstrated, for the first time, that diacerein suppresses the increase in plasma NO level during the development of rat adjuvant-induced arthritis. The therapeutic effects of diacerein on osteoarthritis and those observed in several animal models may be due, at least in part, to the inhibitory effect of rhein, an active metabolite, on NO production in articular chondrocytes.

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