Antiarthritic Effects of Relaxin, in Combination with Estrogen, in Rat Adjuvant-Induced Arthritis

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ABSTRACT

The incidence and severity of rheumatoid arthritis (RA) are reduced during pregnancy. Estradiol-17β and relaxin (RLX), hormones of pregnancy, are implicated in decreased immune responsiveness. The aim of this study was to determine the effects of estrogen and RLX, alone or in combination, on the development of adjuvant-induced arthritis (AIA) in ovariec-to-mized (OVX) Lewis rats. Arthritis was induced on day 0 by adjuvant injection in the left hind paw. Rats were treated with estradiol valerate (E), porcine RLX, E + RLX, or vehicle. Healthy OVX control animals were used for comparison. Treatment with RLX or E alone decreased adjuvant-induced inflammation in both the injected (primary) and noninjected (secondary) hind paws. Combined treatment with E and RLX was more effective than either hormone alone in blocking secondary paw inflammation. Furthermore, E plus RLX reduced changes to spleen and thymus weights induced by adjuvant injection. Both E and RLX alone decreased circulating tumor necrosis factor (TNF) α. The combination of E and RLX resulted in a greater decline in TNFα than treatment with either hormone alone. There was no effect of hormones on the proinflammatory cytokine, interleukin (IL)-1β. The anti-inflammatory cytokine IL-10 increased in response to E and E plus RLX. In conclusion, combined therapy with E and RLX was more effective than either hormone alone in reducing chronic inflammation, joint changes, and high circulating TNFα associated with AIA in rats. Accordingly, these hormones could play a role in reducing RA-induced inflammation during pregnancy by an effect on the immune system.

Rheumatoid arthritis (RA) affects upwards of 2 million people in the United States and is two to three times more prevalent in women (Wood, 2004). It is an autoimmune disease typified by white blood cell infiltration of the synovium resulting in inflammation that leads to cartilage destruction and bone loss (Cutolo and Straub, 2000). Symptoms of RA and other T-helper cell type 1 (Th1) autoimmune diseases (Miossec and van den Berg, 1997) diminish during pregnancy (Förger et al., 2005) and/or with the use of oral contraceptives (Doran et al., 2004).

Estrogens, elevated during pregnancy, reduce inflammation in a mouse model of autoimmune arthritis (Latham et al., 2003) and in a rat model of adjuvant-induced arthritis (Badger et al., 1999). Relaxin (RLX), another hormone of pregnancy, remodels connective tissue (Unemori et al., 1993; Lenhart et al., 2001) and has been reported to play a role in wound healing (Unemori et al., 2000) and modulation of immune function (Piccinni et al., 1999). Relaxin also inhibits activation of neutrophils stimulated by proinflammatory agents in vitro (Masini et al., 2004). This down-regulation of neutrophil function might account for the RLX-induced reduction in inflammation reported in ischemia/reperfusion models (Bani et al., 1998). RLX can act in a manner similar to estrogen. For example, RLX promotes uterine growth and can activate estrogen receptor signaling pathways (Yan et al., 2006), and pretreatment with estrogen enhances RLX action in ovariectomized rodents (Vasilenko et al., 1980). Studies in human THP-1 macrophage cells showed that RLX reduces endotoxin-induced inflammatory cytokine production (Dschietzig et al., 2004). Relaxin also stimulates leukocyte adhesion and migration in studies using THP-1 cells and peripheral blood mononuclear cells (Figueiredo et al., 2006). There are no reported studies on the effect of RLX on RA; however, RLX could be important in the reported pregnancy-related improvements in RA by reducing production of proinflammatory cytokines.

Described by Pearson in 1956, rat adjuvant-induced arthri-
tis (AIA) is an autoimmune model of RA characterized by a systemic inflammation of peripheral joints. The model is similar to chronic human inflammatory arthritis with respect to clinical symptoms and histopathology (Gugasyan et al., 1997) and is considered to be an important experimental model of chronic inflammation related to RA. Cytokines play an important role in the inflammation cascade, and changes in these proteins can be monitored to determine how a given treatment is altering disease progression. Some of the important proinflammatory cytokines involved in RA are interleukin (IL)-1β, tumor necrosis factor (TNF) α (Feldmann et al., 1996), and vascular endothelial growth factor (VEGF), an angiogenic cytokine (Han et al., 2004). Anti-inflammatory cytokines such as IL-10 are also of interest in RA research (Bach, 1996). As an extension of work reported earlier (Santora et al., 2005) and given the role of RLX in tissue remodeling and wound repair, the objective of this study was to determine the effects of RLX and estrogen, alone and in combination, on the development of AIA in the rat through analysis of joint inflammation and destruction, tissue changes, and circulating cytokines.

Materials and Methods

Materials. 17-β-Estradiol valerate was obtained from ICN Biomedicals (Aurora, OH). Purified porcine RLX was prepared by extraction and purification from ovaries of pregnant sows (Sherwood and O'Byrne, 1974). Benzopurpurin (BZ; Acros Organics, Morris Plains, NJ) was used to facilitate the slow release of porcine RLX over a 24-h period (Steinetz et al., 1960). Bovine serum albumin (BSA) and sesame oil were obtained from Sigma (St. Louis, MO). Phosphate-buffered saline (PBS) was acquired through Invitrogen (Carlsbad, CA). Xylazine from Phoenix Pharmaceuticals Inc. (St. Joseph, MO) and ketamine from Dodge Animal Health (Fort Dodge, IA) were used for anesthesia. The Mycobacterium butyricum for adjuvant was obtained through Difco Laboratories (Detroit, MI), and light mineral oil was from Fisher Scientific (Fairlawn, NJ). The following rat QuantiKine ELISA kits were purchased from R&D Systems (Palo Alto, CA): TNFα, IL-1β, VEGF, and IL-10.

Animals. All animal procedures were approved by the Merck and Co., Institutional Animal Care and Use Committee in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Animals. Ovariectomized (OVX) Lewis rats were used for anesthesia. The Mycobacterium butyricum for adjuvant was obtained through Difco Laboratories (Detroit, MI), and light mineral oil was from Fisher Scientific (Fairlawn, NJ). The following rat QuantiKine ELISA kits were purchased from R&D Systems (Palo Alto, CA): TNFα, IL-1β, VEGF, and IL-10.

Adjuvant-Induced Arthritis. On day −10, rats were weighed and earmarked for identification. Three days before paw injections, rats were tattooed (AIMS, Hornell, NY) directly above both ankle joints to provide an anatomical marker to minimize variability when measuring paw volume. Adjuvant was prepared by grinding M. butyricum in a mortar and mixing with light mineral oil to a final concentration of 5 mg/ml. On day 0, rats were anesthetized using a combination of xylazine (13 mg/kg) and ketamine (87 mg/kg i.m.) in the hind leg. Arthritis was induced by a subplantar injection of adjuvant (0.1 ml) in the dorsum of the left hind paw between the third and fourth digits. The volume of both hind paws was measured on day 0 (before adjuvant injection) and then weekly using mercury displacement plethysmography (Buxco, Wilmington, NC). Change in paw volume was calculated as the difference between volumes on day 0 and volumes recorded on days 7, 14, and 21. Body weights were recorded on days 0, 7, 14, and 21. Animals were euthanized by carbon dioxide inhalation on day 21. This animal model was chosen because it exhibits a rapid primary inflammation response to the adjuvant followed by a secondary systemic immune response (Fletcher et al., 1998). In this way, the hormones could be evaluated for their effects on both phases of the model.

Treatments. In this design, adjuvant-injected rats were divided into four groups (n = 8 rats/group) and treated with 17-β-estradiol valerate (E; 5 μg/0.1 ml sesame oil s.c., weekly starting day −10), porcine RLX (8 μg/0.1 ml 1% BZ/0.1% BSA in PBS s.c., daily beginning days −3 through 21), E + RLX, or vehicle (V; 0.1 ml sesame oil s.c., weekly; and 0.1 ml 1% BZ/0.1% BSA in PBS s.c., daily). A group of healthy OVX control rats (n = 8, no adjuvant/no treatment) were included for comparison. The dose, route, and timing of RLX administration were chosen to simulate circulating mid- to late gestation levels of RLX in adult female rats (Bani et al., 2001). The dose of E used was based on studies showing that estrogen treatment enhances RLX responsiveness in ovariectomized rats (Vasilenko et al., 1980).

Radiographic Analysis and Organ Weights. On day 21, hind limbs were subjected to radiographic analysis using a Faxitron X-ray machine (Hewlett-Packard, Buffalo Grove, IL) with a 0.5-mm focal spot, beryllium window, and X-OMAT TL (nonscreen) film. The focal film distance was 61 cm, and exposures were made over 30 s at 45 kVp and 3 mA. Radiographs were analyzed by a board-certified radiologist who was blinded to the treatment groups. Quantitative scores were generated for radiographic changes in the joints in the following areas: soft tissue volume, joint space, subchondral erosion, periostitis, osteosclerosis, subluxation, and degenerative joint changes. The values were based on increasing severity in which 26 was the highest possible score per paw (Fletcher et al., 1998). Spleen and thymus were harvested from each rat for wet weight to detect splenomegaly and thymic involution typical of AIA (Fletcher et al., 1998).

Cytokine Analysis. After euthanasia, blood was collected by cardiac puncture into heparinized Vacutainers. Blood samples were centrifuged at 1360 rpm (400g at 4°C for 6 min), and the plasma was stored at −80°C. The concentration of TNFα, IL-1β, VEGF, and IL-10 in rat plasma samples was measured using Quantikine rat immunoassay kits.

Statistical Analysis. Data were analyzed by analysis of variance and tested for differences using Fisher’s least significant difference test for multiple comparisons (Excel Analyze-it Software, Leeds, UK). Organ weight and circulating cytokine results are shown as scattergrams in which each point represents an animal, and the mean is indicated by a line. Values in the text are expressed as the mean ± S.E.M., and p < 0.05 was accepted as significant.

Results

Paw Volume Changes in Adjuvant-Induced Arthritis. Healthy OVX rats showed little paw volume change over the course of the study (Fig. 1). Adjuvant injection resulted in progressive swelling of the injected (primary) hind paw that increased over time up to day 21. Treatment of adjuvant-injected rats with hormones (E, RLX, or E + RLX) did not influence paw volume by day 7. However, by day 14, all hormone treatments decreased primary paw inflammation compared with vehicle controls (p < 0.05), and this reduction in primary paw volume continued to day 21 (p < 0.05).

Figure 2 illustrates that a secondary inflammation of the noninjected hind paw was evident in vehicle controls by day 14 of the study. Paws of rats treated with RLX or E + RLX were less swollen than vehicle controls on day 14. By day 21, rats treated with RLX alone showed a trend toward decreased secondary paw inflammation compared with V
animals had a mean score of 0. Weekly treatment with E in combination with daily RLX administration in adjuvant-injected rats reduced arthritic changes in primary (8.7 ± 1.4) and secondary (5.6 ± 1.4) paws compared with arthritic vehicle controls (p < 0.05; Fig. 3C).

**Body and Organ Weights of Animals Injected with Adjuvant and Subjected to Hormone Treatment.** Adjuvant-injected rats weighed substantially less on day 21 (177.5 ± 4.8 g) compared with healthy control animals (214.6 ± 5.3 g, p < 0.05; Fig. 4A). Treatment with E alone further reduced body weight compared with arthritic controls (E = 144.3 ± 2.1 g, p < 0.05). RLX treatment alone had no effect on the body weight of adjuvant-injected rats compared with vehicle controls. However, adjuvant-injected rats treated with RLX weighed more than rats treated with E alone (RLX = 184.0 ± 3.5, p < 0.05). The combination of E + RLX significantly mitigated the E-induced body weight loss (E + RLX = 157.9 ± 5.1 g, p < 0.05).

Adjuvant injection resulted in a marked increase in spleen wet weight (0.82 ± 0.06 g) compared with healthy O VX animals (0.50 ± 0.01 g, p < 0.05; Fig. 4B). Although RLX alone had no effect on adjuvant-induced splenomegaly, treatment with E alone (0.57 ± 0.03 g) or E + RLX (0.51 ± 0.03 g) resulted in decreased spleen weights compared with vehicle controls (p < 0.05). These weights were comparable with spleen weights of healthy animals (0.50 ± 0.01 g). By day 21, thymus weight in adjuvant-injected animals (0.25 ± 0.03 g) was markedly lower in comparison with healthy control rats (0.80 ± 0.03 g, p < 0.05; Fig. 4C). Treatment with E increased thymic involution in adjuvant-injected rats (0.11 ± 0.01 g) in comparison with vehicle-treated controls (p < 0.05), whereas RLX alone or in combination with E had no effect on thymus weight.

**Circulating Cytokine Levels on Day 21 in Rats Subject to Adjuvant Arthritis.** Adjuvant-injected, vehicle control rats showed an increase in the proinflammatory cytokine TNFα on day 21 of the study (946.5 ± 21.9 pg/ml; Fig. 5A)
relative to healthy control animals (619.9 ± 5.8, p < 0.05). Weekly E treatment and daily RLX administration each were effective alone in reducing systemic TNFα (E = 716.4 ± 22.0 pg/ml, RLX = 704.1 ± 25.3 pg/ml) compared with vehicle controls (p < 0.05). When administered in combination, E + RLX were more effective than either hormone alone in reducing circulating TNFα (p < 0.05) to a level close to that of healthy controls (646.6 ± 8.4 pg/ml). Circulating levels of the proinflammatory cytokine IL-1β in healthy control rats (158.6 ± 6.1 pg/ml) increased in response to adjuvant injection (277.5 ± 22.8 pg/ml, p < 0.05). Treatment of adjuvant-injected animals with E, RLX, or E + RLX had no effect on systemic IL-1β (data not shown).

Circulating levels of VEGF, an indicator of angiogenesis, were higher in adjuvant-injected animals (204.9 ± 8.6 pg/ml) compared with healthy controls (157.0 ± 3.0 pg/ml, p < 0.05; Fig. 5B). Treatment of adjuvant-injected rats with E or RLX alone had no effect on circulating VEGF. However, arthritic rats treated with E in combination with RLX had higher circulating VEGF (257.4 ± 15.5 pg/ml, p < 0.05) compared with vehicle controls and arthritic rats treated with E or RLX alone. Healthy OVX rats had low circulating levels of the anti-inflammatory cytokine IL-10 (19.2 ± 0.4 pg/ml; Fig. 5C) in comparison with adjuvant-injected animals (40.2 ± 5.7 pg/ml, p < 0.05). Adjuvant-injected rats treated with E (51.3 ± 5.1 pg/ml) or E + RLX (61.0 ± 10.4 pg/ml), but not RLX alone, had higher IL-10 levels compared with arthritic vehicle controls (p < 0.05).

**Discussion**

To determine whether the hormone RLX, in concert with E, was effective in reducing markers of inflammation, these
hormones were evaluated, alone and in combination, in a rodent model of RA (Fletcher et al., 1998). Estrogen has known anti-inflammatory effects in models of arthritis (Badger et al., 1999; Latham et al., 2003), and those effects were also observed in this study with respect to paw swelling and circulating cytokine levels. In addition, weekly treatment with E also resulted in thymic involution and increased body weight loss compared with controls. Treatment with RLX or E alone was equally effective in decreasing adjuvant-induced joint inflammation in the injected paw and systemic TNFα. Moreover, combined therapy of weekly E with daily RLX was more effective than either hormone alone in reducing paw swelling and circulating TNFα but also mitigated the side effects of body weight loss and increased thymic involution observed with E alone.

In the rat AIA model, the adjuvant-injected paw was typified by a rapid onset of inflammation evident within 24 h of adjuvant injection and continued to increase up to day 21 (Fletcher et al., 1998). All hormone treatments (E, RLX, and E + RLX) were effective in reducing this primary edema by day 21 compared with arthritic controls. The noninjected paw was subject to a secondary inflammation by day 14 post-adjuvant injection as a result of the immune response to the bacterial adjuvant (Pearson, 1956). Treatment of adjuvant-injected rats with RLX alone showed a strong trend toward reduced secondary paw inflammation. However, because this secondary inflammation is the indirect result of an autoimmune effect (Lussier et al., 1981), the paw volumes were more variable within a group. Nevertheless, combined treatment with E and RLX was effective in reducing secondary paw inflammation, and these results were confirmed by radiographic analysis of the soft and hard tissue changes in the joints (Santora et al., 2005). The effects of estradiol-17β in reducing paw swelling and bone mineral density in a rat model of AIA have been reported (Badger et al., 1999). Although there is evidence for therapeutic benefits of RLX in fibrosis (Unemori et al., 1993) and wound healing (Unemori et al., 2000), there is no information on the efficacy of RLX in models of arthritis. Since many anti-rheumatic drugs have been evaluated using the rat AIA model, the effects of E in combination with RLX described here were compared with the reported effects of immunosuppressive (e.g., dexamethasone) and anti-inflammatory agents (e.g., indomethacin) on rat AIA. Dexamethasone, a potent glucocorticoid and anti-rheumatic compound, decreases primary and secondary paw inflammation by almost 100% in the rat AIA model; however, body weight is severely reduced (Theisen-Popp and Muller-Peddinghaus, 1994). Treatment of male, AIA rats with indomethacin reduced both primary, injected paw (57%) and secondary, noninjected paw (66%) inflammation compared with untreated arthritic rats (Theisen-Popp and Muller-Peddinghaus, 1994). In addition, folate-targeted immunotherapy in female AIA rats resulted in a >60% reduction in swelling of noninjected paws compared with arthritic controls (Paulos et al., 2006). In the current studies, comparable anti-inflammatory effects of E in combination with RLX were observed in OVX, Lewis rats with AIA shown by inhibition of both primary (48%) and secondary (62%) paw swelling compared with arthritic controls.

A characteristic of AIA is marked body weight loss by day 21 (Fletcher et al., 1998). Ovariectomized, adjuvant-injected rats treated with E lost more body weight compared with rats only injected with adjuvant. This is consistent with studies that indicate estrogen reduces meal size and food intake in OVX rats (Geary and Asarian, 1999). Moreover, in OVX rats subject to an acute inflammatory challenge, anorexia and weight loss were more severe with E treatment (Lennie, 2004). The weight loss observed in the E-treated AIA rats in the present study was attenuated by coadministration of RLX. The explanation for this is unclear. However, RLX is reported to increase drinking behavior in late pregnant rats (Summerlee et al., 1998). A RLX-induced increase in water consumption could account for the prevention of body weight loss in E + RLX-treated rats. Thymic involution and splenomegaly also are hallmarks of AIA (Pearson, 1956). In the present study, E increased thymic involution and reduced splenomegaly. These results are consistent with data implicating increased estrogen as a cause of pregnancy-induced thymic involution (Tibbetts et al., 1999). Likewise, estrogen reduces splenomegaly in a murine model of lupus (Schoenroth et al., 2004). Other studies indicate that removal of estrogen by ovariectomy results in enlargement of the thymus (Oner and Ozan, 2002). There is no evidence for a direct effect of RLX on rat spleen or thymus. The RLX receptor, LGR7, is not expressed in rat spleen (Hsu et al., 2000), which could explain the lack of a RLX effect on adjuvant-induced splenomegaly. Whether the rat thymus expresses LGR7 is unknown.

The proinflammatory cytokine TNFα is a systemic marker of inflammation (MacNaul et al., 1990). All hormone treatments reduced circulating TNFα in adjuvant-injected rats, and the combined treatment with E + RLX reduced plasma TNFα to the level detected in the circulation of healthy OVX rats. Estrogen treatment reduces TNFα and other cytokines produced by Th1-type lymphocytes (Saleem, 2004). In addition, low physiologic levels of RLX reduce TNFα production in THP-1 cells challenged with LPS to create an inflammatory response (Dschietzig et al., 2004). However, this is the first report that RLX is effective alone, and in combination with E, in suppressing high circulating TNFα in vivo. Systemic levels of TNFα and IL-1β are suppressed during pregnancy (Schuna, 2002) and elevated in patients with RA (Feldmann et al., 1996). In rat AIA, both E and RLX administration reduced high systemic TNFα but had no effect on elevated IL-1β. The explanation for the differential effect of these pregnancy hormones on these two Th1-type cytokines in this study is unknown. In human THP-1 macrophage cells, RLX decreases endotoxin-stimulated IL-1 and TNFα secretion (Dschietzig et al., 2004), so the inability of RLX to inhibit IL-1β in vivo was unexpected. Studies show that both estrogen (Latham et al., 2003) and RLX (Piccinni et al., 1999) reduce the Th1 immune response. The absence of an effect of either hormone on circulating IL-1β reported here may be a result of the timing of cytokine measurement relative to treatment and/or the experimental model used (Latham et al., 2003).

All adjuvant-injected animals had higher levels of circulating VEGF compared with controls, which was expected because increased vascularity is a marker of inflammation (Han et al., 2004). Treatment with E in combination with RLX resulted in the greatest increase in circulating VEGF, consistent with the reported activity of RLX increasing VEGF expression during wound healing (Unemori et al., 2000). The decreased paw swelling in the E + RLX animals
is the net effect of these hormones on multiple immune mediators. Thus, the decline in proinflammatory TNFα and increase in anti-inflammatory IL-10 suggests that both pro- and anti-inflammatory cytokines are involved in the decreased paw swelling observed in response to combined hormonetreatment. In summary, there were significant effects of E and RLX, alone and in combination, in this model of rat AIA. Treatment of adjuvant-injected OVX rats with RLX served to reduce injected paw inflammation and circulating TNFα compared with arthritic controls. These in vivo data extend observations in vitro that showed that RLX, administered at low doses, decreases endotoxin-induced TNFα production by THP-1 cells (Dschietzig et al., 2004). Treating adjuvant-injected rats with E resulted in a reduction of injected paw volume, splenomegaly, and circulating TNFα levels, as well as an increase in the anti-inflammatory cytokine IL-10 compared with vehicle controls. These results confirm previous reports of the anti-inflammatory effects of estrogen in models of arthritis with respect to paw swelling and proinflammatory cytokines (Badger et al., 1999; Latham et al., 2003). However, E-treated rats suffered from considerable weight loss and increased thymicinvolution. The combined treatment of weekly E with daily RLX resulted in decreased swelling in both injected and noninjected paws. With this combined therapy, the animals also demonstrated decreased splenomegaly and reduced TNFα, in addition to increased circulating IL-10. Moreover, the combination treatment mitigated the weight loss and thymicinvolution seen with E alone. These data emphasize the importance of studying the effects that hormones of pregnancy, such as estrogen and RLX, have on autoimmune disease remission, including RA. Although RLX has long been known as a hormone of pregnancy, multiple roles for this family of hormones are emerging with the discovery of new RLX family genes and the evidence for RLX receptors in nonreproductive tissues (Hsu et al., 2000) with the potential for novel clinical targets. Whether RLX could be used therapeutically to reduce symptoms of RA in patients is unknown. Further research to understand the mechanism of action of the anti-inflammatory and jointprotective effects of RLX, in combination with E, is needed. However, the reduction in circulating TNFα in the rat AIA model suggests an effect on immune function that has implications in the development of novel therapies for the treatment of rheumatoid arthritis.

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References


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