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 ovariectomized (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 twice as common 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 (Vasilienko 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 (Figuerido 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 DiCo 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 obtained from Charles River Laboratories (Portage, MI) at 6 weeks of age and were housed in groups of three or four in Tecniplast 1291 rodent boxes (Exton, PA) under specific pathogen-free conditions. OVX rats were used so that circulating estrogen levels could be controlled during the study. Rats were acclimatized for 1 week before the study. Rooms were maintained at 21°C and 50% relative humidity with a 12-/12-h light/dark cycle. Rats were provided with Harland 888 Santora et al.

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
and 9.6 ± 1.3 for the secondary paw, whereas healthy control 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 auto-immune response (Lussier et al., 1981), the paw volumes were more variable within a group. Nevertheless, combined treatment with E and RLX was equally effective in decreasing adjuvant-induced 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.

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 (Salem, 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 vascularization 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 hormone treatment.

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 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 estrogens 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 thymic involution. 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 thymic involution 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 joint protective 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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