Effects of Melittin on the Production of Matrix Metalloproteinase-1 and -3 in Rheumatoid Arthritic Fibroblast-Like Synoviocytes

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Abstract. Bee venom (BV) has been used in patients with rheumatoid arthritis, a condition characterized by rheumatoid joint destruction mediated, in large part, by matrix metalloproteinases (MMPs). We investigated the effects of melittin, a major component of bee venom, on the production of MMPs in human rheumatoid arthritic fibroblast-like synoviocytes (FLS). Lipopolysaccharide (LPS)-stimulated MMP3 production was significantly inhibited by melittin, which also inhibited LPS-induced DNA binding by nuclear factor κB (NF-κB). Mellitin had no effect on IL-1β- or TNF-α-induced MMP1 or MMP3 production and did not decrease LPS-induced secretion of MMP1. Taken together, these findings suggest that melittin may exert its anti-rheumatoid effects, at least in part, by inhibiting MMP3 production, most likely through inhibition of NF-κB activity.

Keywords: rheumatoid arthritis, melittin, matrix metalloproteinase-1 and -3

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by inflammatory cell infiltration, synovial tissue hyperplasia, and a progressive destruction of cartilage and bone (1). Fibroblast-like synoviocytes (FLS), described as transformed or tumor-like in appearance, or simply activated, are believed to play a key role in the pathogenesis of RA (2). In addition to increased levels of adhesion molecules, FLS typically express and secrete matrix-degrading enzymes, which cleave extracellular matrix scaffolding and allow FLS to invade surrounding tissue. The expression of matrix metalloproteinases (MMPs), which can degrade a wide variety of ECM components, including fibrillar collagens (3), is positively regulated by the well-characterized pro-inflammatory chemokines, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and lipopolysaccharide (LPS). MMPs, considered the most important matrix-degrading enzymes in RA, act as key mediators of the resorption of cartilage, bone, synovial fluid, and adjacent soft tissue that occurs as part of the pathological destruction of joint tissue (4). MMP1, also known as collagenase 1, is one of the primary enzymes responsible for the degradation of type II collagen (5). MMP3 (stromelysin 1) is active against cartilage matrix components, such as proteoglycan and fibronectin, and can activate pro-MMPs (6).

Bee venom (BV) has been used in patients with arthritis, including rheumatoid arthritis, to reduce pain and edema (7). In the arthritic rat model, BV has been shown to act as an anti-inflammatory adjuvant (8) and to reduce LPS-induced inflammatory edema and polyarthritis (9). The hydrophilic peptide melittin, the major active ingredient of BV, may retain anti-RA activity in rabbit models of immune-mediated arthritis (10). We have previously shown that melittin down-regulates MMP-3 expression in human osteoarthritic chondrocytes (11). Here, we examined the effects of melittin on MMP1 and MMP3 production in human FLS.

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Melittin Inhibits MMP-3 Production in RA FLS obtained from RA patients.

Synovial tissue samples were obtained at the time of total knee joint replacement from 15 patients with longstanding RA (mean age, 65.0 ± 21.3 years; mean disease duration, ≥10 years). RA patients had active disease, as evidenced by pauciarticular joint involvement (≥3 tender and/or swollen joints) and an increase in acute-phase reactants (mean serum C-reactive protein level, 21 ± 16 mg/l). Eleven patients were positive for rheumatoid factor IgM. At the time of surgery, patients presented with symptomatic disease requiring treatment with NSAIDs or selective COX-2 inhibitors. Patients who had received intra-articular steroid injections were excluded. Synovial tissue samples, collected in sterile PBS, were chopped and digested with collagenase for 2 h at 37°C. After removing tissue debris by filtering through a 70-µm cell strainer (BD Falcon™, Bedford, MA, USA), cells were re-suspended in growth media containing DMEM plus 4.5 g/l glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% FBS in a humidified, 37°C incubator with ambient oxygen and 5% CO₂. Cells were maintained under subconfluent conditions and used between passages three and five.

For tests of cell viability after exposure to melittin, FLS were seeded at 2 × 10⁵ cells/well in 96-well culture plates; treated with different doses of melittin (1, 10, 100, 500, 1000, 5000, and 10000 ng/ml) and incubated for 4, 12, 24, and 48 h. FLS viability was measured using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT)-based assay kit (Roche, Basel, Switzerland). A 50-µl aliquot of XTT solution was added, and after incubating for 5 h, absorbance was measured at an excitation wavelength of 450 nm and an emission wavelength of 630 nm. Three independent experiments were performed in triplicate.

MMP expression in patient samples was evaluated by Western analysis of FLS extracts prepared according to standard procedures. Proteins were separated by SDS-PAGE using 10% gels (Bio-Rad, Hercules, CA, USA) and transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Freiberg, Germany) using a Mini Trans-Blot apparatus (Bio-Rad). After blocking for 1 h, membranes were incubated overnight at 4°C with rabbit anti-human polyclonal antibodies (diluted 1:200) against MMP-1 or -3 (R&D systems, Minneapolis, MN, USA). The membranes were then washed with PBS containing 0.05% Tween 20 and incubated with an alkaline phosphatase-conjugated antibody (Chemicon, Temecula, CA, USA) for 1 h. Protein bands were detected using enhanced chemiluminescent reagents (Amersham Pharmacia Biotech, Uppsala, Sweden).

The concentrations of MMP1 and MMP3 in conditioned media were determined using commercially available ELISA kits (R&D Systems) according to the manufacturer’s instructions. In brief, 10⁶ cells/well were

![Fig. 1](image-url)

**Fig. 1.** Effects of melittin on the viability of human RA FLS and on LPS-, IL-1β-, and TNF-α-stimulated MMP1 and MMP3 protein expression. A: The viability of melittin on the FLS was evaluated using an XTT cell proliferation assay kit and expressed as the percentage of viable cells relative to the control. Data are presented as mean ± S.D. of three independent experiments performed in triplicate. *P<0.05 vs control. B: Cells were activated with LPS (1 µg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for 2 h prior to exposure to melittin (1, 10, and 100 ng/ml) for 24 h. Culture media were collected and MMP1 and MMP3 levels were determined by Western blot analysis. NC = negative control, PC = positive control.
added to each well of a 12-well plate, serum starved for 2 h prior to treatment with the appropriate reagents, and then incubated for 24 h. Absorbance of supernatants, measured using a microplate spectrophotometer, was used to calculate the concentrations of MMP1 and MMP3 by reference to a standard curve. Four separate ELISA experiments were conducted; each condition was tested in triplicate.

Statistical analyses were performed by using Student’s t-tests and one-way analysis of variance (one way-ANOVA). A value of \( P<0.05 \) was considered significant. Data are presented as means ± S.D.

The dose dependence of melittin cytotoxicity is depicted in Fig. 1A. No significant difference in viability was observed at concentrations up to 500 ng/ml melittin and incubation times as long as 48 h. Significant cytotoxic effects were observed at a melittin concentra-

tion of 1000 ng/ml after 4-h incubation. Based on these data, melittin concentrations of 1, 10, and 100 ng/ml were employed in subsequent experiments.

To evaluate the effects of melittin on the production of MMP1 and MMP3, we measured MMP1 and MMP3 protein levels in LPS-, IL-1β-, or TNF-α-activated FLS by Western analysis. Our results confirm that each of these agents can stimulate MMP production in FLS. Treatment with increasing concentrations of melittin (1, 10, and 100 ng/ml) for 24 h resulted in a significant decrease in MMP1 and MMP3 production in LPS-activated FLS (Fig. 1B). Melittin had no effect on MMP1 and MMP3 production in either IL-1β or TNF-α–activated FLS. To confirm these results, we measured MMP1 and MMP3 levels in conditioned media by ELISA (Fig. 2: A and B). Consistent with the results of immunoblot analysis, melittin treatment attenuated

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**Fig. 2.** Effects of melittin on LPS-, IL-1β–, and TNF-α–stimulated MMP1 and MMP3 secretion in human RA FLS. After cells were cultured, serum-starved FLS were stimulated with LPS (1 µg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for 2 h and then treated with melittin (1, 10, and 100 ng/ml). After 24 h, culture media was collected and analyzed for MMP1 and MMP3 by ELISA. Each column represents the mean ± S.D. of four separate experiments performed in triplicate. *\( P<0.05 \), **\( P<0.01 \) vs control.
Melittin Inhibits MMP-3 Production in RA FLS

LPS-induced MMP3 production measured as secreted protein. Moreover, the inhibitory effects of melittin were dose-dependent, with 100 ng/ml melittin producing the greatest inhibitory effect. MMP1 production, however, was only decreased at the highest melittin concentration (100 ng/ml). Again, melittin had no effect on MMP1 or MMP3 production in either IL-1β- or TNF-α-activated FLS.

Nuclear factor kappa B (NF-κB) is a transcription factor that has been shown to promote the expression of pro-inflammatory cytokines, chemokines, nitric oxide (NO), TNF-α, and IL-6 in RA in response to extracellular signals. To determine whether the inhibitory effect of melittin is dependent on signal transduction pathways involving NF-κB, we used electrophoretic mobility shift assays (EMSA) to assess melittin-dependent changes in NF-κB DNA-binding activity. As expected, LPS induced an increase in NF-κB DNA-binding in FLS. Treatment with melittin attenuated NF-κB DNA-binding activity compared to control samples, producing maximal effects at 4 h. Supershift experiments using antibodies to the NF-κB subunits, p50 and p65, showed an increase in the electrophoretic mobility of radiolabeled bands, confirming that the observed DNA binding was NF-κB specific (Fig. 3).

These observations demonstrate that melittin inhibits the NF-κB pathway, and it likely acts through this mechanism to suppress MMP3 expression.

The aim of the present study was to gain a better understanding of the therapeutic effects of melittin on RA patients. We have previously shown that BV acts through caspase-3 activation to induce apoptosis in human rheumatoid synovial fibroblasts (12). Others have reported that BV induces apoptosis and inhibits cyclooxygenase-2 mRNA expression in a human lung cancer cell line (13). Melittin, specifically, has been shown to exert anti-inflammatory effects by inhibiting LPS-induced expression of cyclooxygenase-2, cytosolic phospholipase A₂, and inducible NO synthase and by modulating intracellular calcium levels and blunting prostaglandin E₂ generation (14).

In the present study, we demonstrate that melittin specifically inhibits LPS-activated MMP3 production. Furthermore, melittin inhibits the LPS-induced DNA-binding activity of NF-κB, consistent with previous reports that melittin prevents LPS-induced NF-κB DNA binding and NF-κB-dependent transcription in FLS by inhibiting I-κB release and p50 translocation (14). Interestingly, we found that melittin does not inhibit TNF-α– or IL-1β–induced MMP3 expression, or NF-κB activity (data not shown). These results are consistent with a recent report that melittin does not decrease NF-κB activity induced by IL-1β or TNF-α (15). Taken together, these results suggest that melittin inhibits LPS-mediated FLS activation by acting on a signaling pathway that LPS does not share with IL-1β or TNF-α. Alternatively, melittin may mediate its anti-inflammatory action through enhanced cortisol secretion, as suggested by a recent report showing stimulation of adrenocorticotropin (ACTH) and beta-endorphin release from the corticotropic cells of the rat adenohypophysis by melittin (16).

In summary, the present study establishes that inhibition of MMP3 production is an integral component of the anti-arthritis effects of melittin, which acts, at least in part, through inhibition of NF-κB.

References