IB-MECA, an A_3 adenosine receptor agonist prevents bone resorption in rats with adjuvant induced arthritis

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Abstract

Objective

The anti-inflammatory effect of adenosine is partially mediated via the A_3 adenosine receptor (A_3AR), a Gi protein associated cell surface receptor. The highly selective A_3AR agonist, IB-MECA was earlier shown to prevent the clinical and pathological manifestations of arthritis in experimental animal models of collagen and adjuvant induced arthritis (AIA). In this study we tested the effect of IB-MECA on the prevention of bone resorption in AIA rats and looked at the molecular mechanism of action.

Methods

Rats with AIA were treated orally twice daily with IB-MECA starting upon onset of disease and the clinical score was evaluated every other day. At study termination the foot, knee and hip region of both vehicle and IB-MECA treated animals were subjected to histomorphometric analysis. Western blot analysis was carried out on paw protein extracts.

Results

IB-MECA ameliorated the clinical manifestations of the disease and reduced pannus and fibrosis formation, attenuated cartilage and bone destruction and decreased the number of osteoclasts. In cell protein extracts derived from paw of AIA rats, A_3AR was highly expressed in comparison to naïve animals. In paw extracts derived from IB-MECA treated AIA rats, down-regulation of the A_3AR protein expression level was noted. PI3K, PKB/Akt, IKK, NF-κB, TNF-α and RANKL were down-regulated whereas caspase 3 was up-regulated.

Conclusion

IB-MECA, a small highly bioavailable molecule, induces modulation of proteins which control survival and apoptosis resulting in the amelioration of the inflammatory process and the preservation of bone mass in AIA rats.

Key words

A_3 adenosine receptor, rheumatoid arthritis, joint damage, PI3K-NF-κB-TNF-α, IB-MECA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease which affects 1% of the adult population. The disease is characterized by hyperplasia of stromal cells and a massive infiltration of hematopoietic cells into the joints , leading to chronic synovitis and destruction of cartilage, bone, tendons and ligaments. Patients with RA show a reduced bone volume and decreased bone turnover, which is further developing to osteoporosis associated with the disease (1). This progressive joint damage results in functional decline and disability (2). About 80% of the affected population becomes disabled within 20 years of symptom onset (3).

The mechanisms responsible for causing joint damage and functional impairment in RA are complicated and involve many pro-inflammatory mediators and degradative enzymes. A member of the TNF family, the receptor activator of NF-κB ligand (RANKL), is required for the differentiation of osteoclasts from their precursor cells, by activation of osteoclastogenesis in inflammatory sites, as well as promoting the osteoclasts activity and survival (4, 5). It is well documented that the bone destruction in RA is mainly mediated by osteoclasts and that RANKL is highly expressed by synovial fibroblasts and T cells in arthritic joints (6). The expression level of RANKL is mediated via the pro-inflammatory tumor necrosis factor-α (TNF-α) known to play an important role in the pathogenesis of RA (7, 8).

Adenosine, which is released into the extracellular environment from activated or metabolically stimulated cells, can bind to selective G-protein-associated membrane receptors, designated A_1, A_2A, A_2B, and A_3 (9). Adenosine plays an important role in limiting inflammation, mainly by preventing the production of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 (10-12).

The highly selective A_3AR agonist IB-MECA, inhibits collagen induced arthritis (CIA) in DBA1 mice, via a mechanism which entails down-regulation of TNF-α and MIP-1a (13). These results have been recently confirmed by our group in the AIA and CIA model utilizing low IB-MECA dosages (10 mg/kg) (14).

In this study we show that in AIA rats, IB-MECA treatment prevents joint damage and bone destruction. In addition we deciphered the molecular mechanism and showed that IB-MECA down-regulates RANKL expression and inhibits the PKB/Akt-NF-κB signal transduction pathway.

Materials and methods

Drugs

IB-MECA was purchased from RBI/Sigma (Natick, MA), and a stock solution of 10 mM was prepared in DMSO. Further dilutions in PBS were performed to reach the desired concentration.

Rabbit polyclonal antibodies against rat, A_3AR and the signaling proteins PI3K, phospho-specific PKB/Akt (S473), IKK a/b, NF-κB, TNF-α, RANKL and cleaved caspase-3 were purchased from Santa Cruz Biotechnology Inc., Ca, USA.

Animal models

Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Kiryat-Matalon, Petach Tikva, Israel. Animals received standardized pelleted diet and tap water ad libitum.

Female Lewis rats, aged 8-10 weeks, obtained from Harlan Laboratories (Jerusalem, Israel), were injected subcutaneously (SC) at the tail base with 100 μl of suspension composed of incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) with 10 mg/ml heat killed *Mycobacterium tuberculosis* (Mt) H37Ra, (Difco, Detroit, USA). Each group contained 10 animals and each experiment was conducted at least three times.

Treatment protocol

IB-MECA was orally administered by gavage, twice daily. The positive controls received vehicle only (DMSO) while the treatment groups received 10 μg/kg of IB-MECA. Treatment was initiated 14 days after immunization.

Clinical disease score

The animals were inspected every second day for clinical arthritis. The scoring system ranged from 0-4 of each limb: 0- no arthritis; 1- redness or swelling of one toe/finger joint; 2- redness and swelling of more than one toe/finger joints, 3-the ankle and tarsal-metatarsal joints involvement. 4- entire paw redness or swelling. The inflammatory intensity was also determined in accordance with the increase in the rat hind paw's diameter, measured by caliper (Mitotoyo, Tokyo, Japan).

Histological score

The foot, knee and hip region of both vehicle and IB-MECA treated animals were collected and fixed in 10% buffered formalin and decalcified in hydrochloric acid (Calci-Clear Rapid) (Pational Diagnostics, Gr, USA) for 24 h. The specimens were then processed for paraffin embedding, histologic 4-μm sections were cut and stained with hematoxylin and eosin. The sections were assessed by a pathologist blinded to the treatment protocols, and each joint was scored separately. The histology score was assessed as follows: A score of 0 to 4 for the extent of inflammatory the infiltration of the cells was used according to the following: 0- Normal; 1 – minimal inflammatory infiltration; 2 – mild infiltration; 3 – moderate infiltration; 4 – marked infiltration. The pannus formation joint tissues, synovial lining cell hyperplasia. The score was graded 0-4: 0-normal; 1- minimal loss of cortical bone at a few sites; 2- mild loss of cortical trabecular bone; 3- moderate loss of bone at many sites; 4- marked loss of bone at many sites with fragmenting and full thickness penetration of inflammatory process or pannus into the cortical bone. The mean of all the histological parameter scores was designated "Histology Score".

Protein extraction from paw

The hind paws were dissected above the ankle joint. The bony tissue was broken into pieces, snap frozen in liquid nitrogen and stored at -80 ºC until use. The paw tissues were added to (4ml/g tissue) RIPA extraction buffer containg 150mM NaCl, 50mM Tris, 1% NP40, 0.5% Deoxycholate and 0.1% SDS. Tissues were homogenized on ice with a polytron, centrifuged and the supernatants were subjected to Western blot analysis.

Western blot analysis

Western blot analyses (WB) of paw extracts were carried out according to the following protocol. Samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50mM Tris buffer pH = 7.5, 150mM NaCl, NP 40). Cell debris were removed by centrifugation for 10 min, at 7500 xg. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 μg) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% BSA and incubated with the desired primary antibody (dilution 1:1000) for 24h at 4ºC. Blots were then washed and incubated with a secondary antibody for 1h

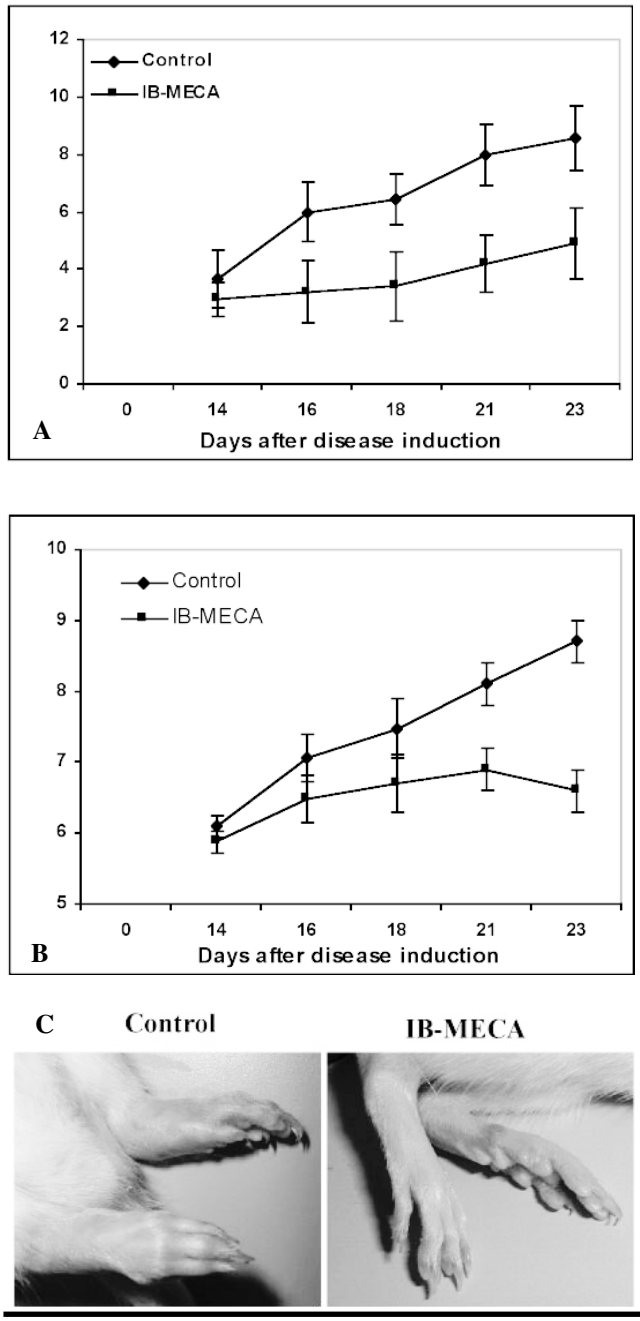


Fig. 1. Effect of IB-MECA on the clinical manifestations of AIA. Rats were immunized with a single imjection of Mycobacterium tuberculosis. Treatment with IB-MECA (10 μg/kg was initiated on day 14th. (**A**) Effect of IB-MECA on disease clinical score. (**B**) Effect of IB-MECA on paw thickness.

at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison,W1, USA). The data presented in the different figures are representative of at least four different experiments.

Statistical analysis

The results were evaluated using the Student's t-test, with statistical significance set at $p < 0.05$. Comparison between the mean value of different experiments was carried out.

Results

Effect of IB-MECA on the development of arthritic score in the AIA model

The clinical signs of the arthritis started to appear on day 14th. In the control group the maximal clinical arthritic score reached a value of 8.7 ± 0.76 whereas in the IB-MECA treated group the maximal score was only 4.8 ± 0.95 (Fig. 1A). In addition, IB-MECA treatment resulted in a 35% ± 1.2 inhibition of the paw thickness (Fig. 1B). The IB-MECA treatment markedly decreased the paw edema. Fig. 1C depicts the severe redness and swelling of the entire paw in the control group (left) in comparison to a representative paw derived from the IB-MECA treated group which appears completely normal (right).

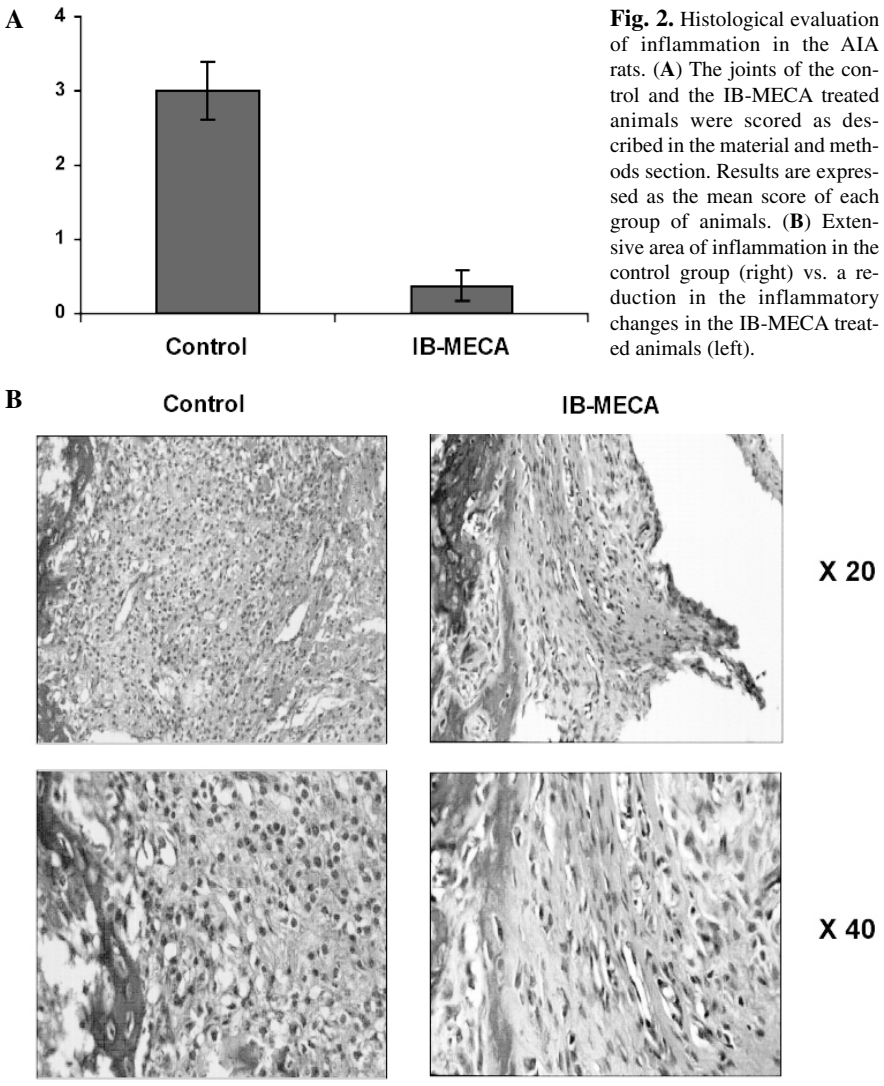


Fig. 2. Histological evaluation of inflammation in the AIA rats. (**A**) The joints of the control and the IB-MECA treated animals were scored as described in the material and methods section. Results are expressed as the mean score of each group of animals. (**B**) Extensive area of inflammation in the control group (right) vs. a reduction in the inflammatory changes in the IB-MECA treated animals (left).

Effects of IB-MECA on the histological features of AIA

On day 23 after disease induction, the animals were scarified and joints from two hind paws of each animal were harvested and examined histologically. The histological analysis was carried out on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage and bone destruction. Most of the histophathological changes were found in the interophalangeal region of the foot. Similar changes in the knee region were noted in the vehicle group while the knee in the IB-MECA treated group remained intact. Overall, the severity of joint histopathology was correlated with the clinical severity index.

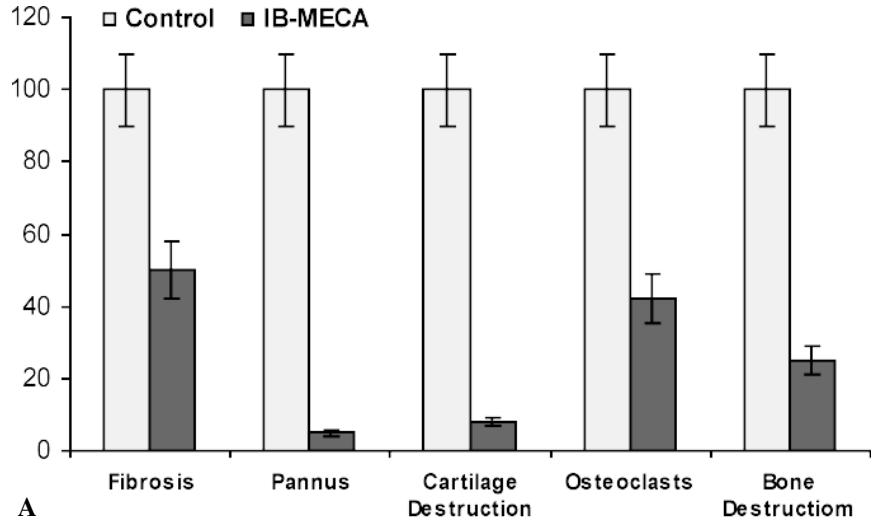
A statistically significant reduction in the inflammatory changes was seen in the joints of treated rats compared to the control group in which extensive area of inflammation was noted (mean total score 0.4 ± 0.034 vs 3.2 ± 0.14, respectively) (Figs 2A and 2B). The synovium in the control group appeared thickened, fibrous, hyperplastic and hypertrophic due to resident synovial cell proliferation and infiltration by mononuclear leukocytes. On the contrary, almost no fibrosis or hyperplasia of the synovia were observed in the IB-MECA treated group (Figs. 3A and 3B).

In the control group massive pannus tissue was present in the destructed areas replacing the normal tissue of the articular space, while in the IB-MECA treated group there was only mild evidence of pannus development (Figs. 3A and 3B). Severe cartilage damage followed by cartilage loss was presented in the control group whereas in the IB-MECA treated animals the cartilage texture appeared to be normal (Figs. 3A and 3B).

A reduction of 73% in the appearance of osteoclasts was noted in the IB-MECA treated group vs. the control group. This was followed by high incidence of bone destruction in the control group. On the other hand, all these parameters were low in the IB-MECA treated animals (Figs. 3A and 3B).

Effects of IB-MECA on the modulation of key signaling proteins down-stream to A_3AR activation in paw extracts derived from AIA rats

Protein extracts were prepared from the hind paws of treated and untreated AIA rats and subjected for WB analysis. Down-regulation of A_3AR was noted upon treatment with IB-MECA, demonstrating that receptor activation and



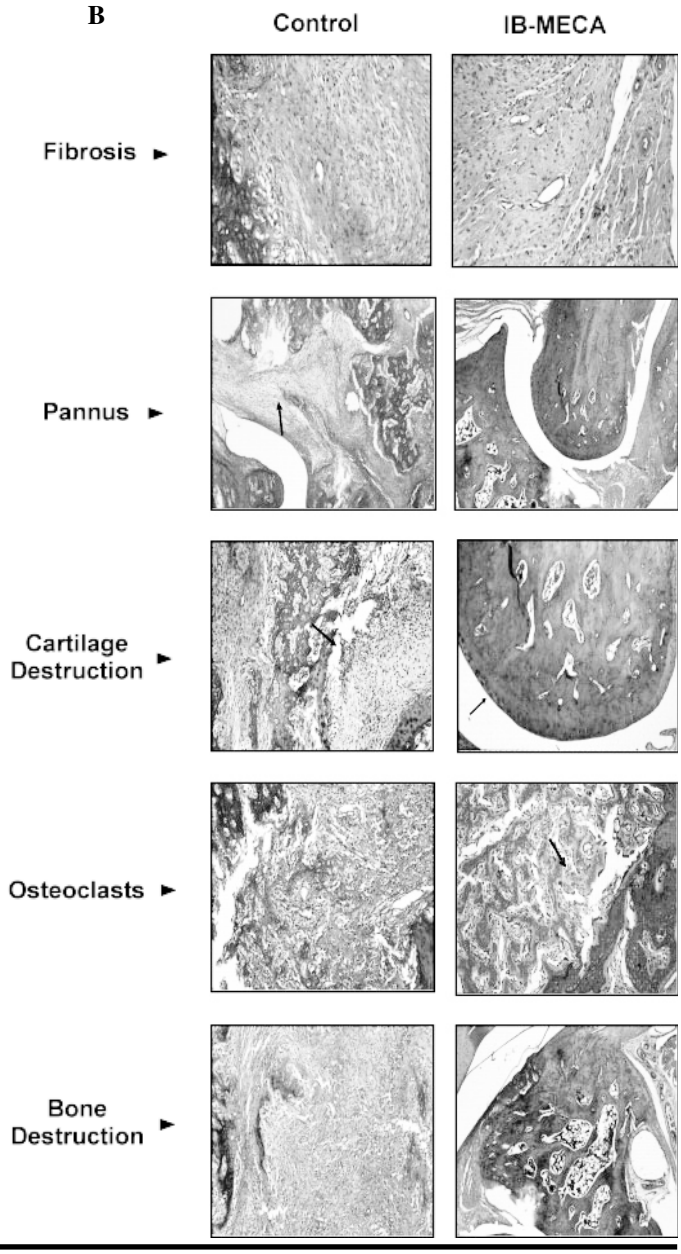


Fig. 3. Histological evaluation of fibrosis in the AIA rats. (**A**) The joints of the control and the IB-MECA treated animals were scored as described in the material and methods section. Results are expressed as the mean score of each group of animals. (**B**) Representative pictures of the joints demonstrating massive fibrosis, large pannus tissue, severe cartilage damage, increased osteoclasts appearance and high incidence of bone destruction in the control group (right) while the morphohistological status in the IB-MECA treated animals appears to be normal (left).

most probably its degradation took place (Fig. 4A). We then looked at key signaling proteins up-stream to TNF-α. A reduction in the protein expression level of PI3K and PKB/AKT was noted in the IB-MECA treated group, in comparison the that of the control followed by down-regulation in the level of the PKB/AKT down-stream kinase, IKK. As a result a reduced level of NF-κB was observed in the IB-MECA treated animals. This chain of events led to a 50% reduction in the expression level of TNF-α upon IB-MECA treatment followed by 40% decrease in the RANKL protein expression level in paw extracts derived from IB-MECA treated AIA rats (Figs. 4B and 4C). In addition, caspase-3 expression level, which is linked to apoptosis, was up-regulated (Fig. 4D).

Discussion

In the present study, IB-MECA was given upon onset of disease and successfully ameliorated inflammation, as well as cartilage and bone resorption.

We first showed that A_3AR is highly expressed in cell extracts derived from paw of AIA rats in comparison to naïve animals. IB-MECA treatment resulted in down-regulation of the A_3AR protein expression level in inflamed paw extracts. This led to the modulation of key signaling proteins involved with disease pathogenesis, resulting in ameliorated intensity of the arthritis and the prevention of bone loss. Subsequently, the histomorphometric observations in this study show that reduced inflammation, pannus and fibrosis took place. Moreover, there was evidence of attenuated cartilage and bone destruction, as well as decreased number of osteoclasts in the IB-MECA treated group.

A balance between osteoclasts resorption and osteoblasts bone formation is essential for maintaining the bone mass in the skeleton (15). Sites of osteoclasts activity have been demonstrated by electron microscopy in areas of erosion in patients with RA, demonstrating the pivotal role of osteoclasts in the pathogenesis of the joint damage (16). There is increasing evidence that cell survival and apoptotic key signaling proteins are involved in the physiological and

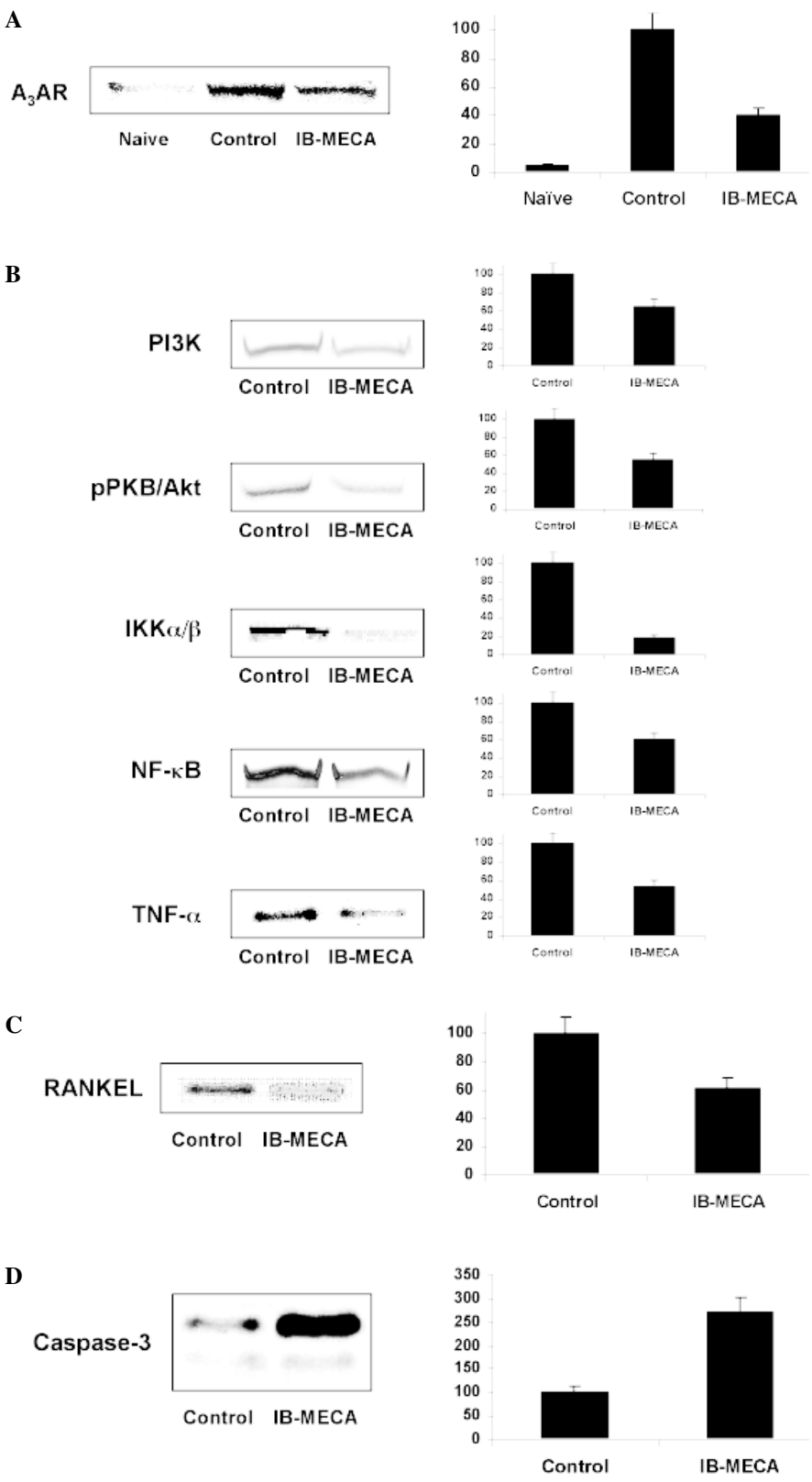


Fig. 4. Effects of IB-MECA on the modulation of key signaling proteins down-stream to A_3AR activation in paw extracts derived from AIA rats. (**A**) A_3AR expression is highly expressed AIA rats in comparison to naïve animals. A_3Ar expression level is down-regulated upon IB-MECA treatment. (**B**). Decrease in the expression level of PI3K, PKB/Akt, IKK, NF-κB and TNF-α is observed upon treatment with IB-MECA. (**C**) Down-regulation of expression level of RANKL is noted in the IB-MECA treated group. (**D**) Up-regulation of caspase-3 expression level is noted in the paw extracts derived from IB-MECA treated animals

pathological processes of osteogenesis. (17, 18). These have prompted us to look at a the pathway of the PI3K - TNF-α known to play a major role in determining survival/apoptosis upon modulation of its key members.

The down-regulation of A_3AR expression upon treatment with IB-MECA observed in the paw protein extracts may represent a response of the cells, populating the joints, to IB-MECA. It has been previously shown that A_3AR is highly expressed in tumor cells and upon receptor activation, receptor down-regulation occurs due to internalization and degradation (19-21). This process, which is typical to other G-protein receptors, is accompanied by generation of down-stream signal transduction pathways. Indeed in this study we observed down-regulation of the PI3K - TNF-α pathway including a decrease in the expression level of PI3K, PKB/Akt, IKK, NF-κB and TNF-α. The down-regulation of TNF-α expression level resulted also in a decreased level of RANKL.

In previous studies we showed that A_3AR activation in melanoma, colon and prostate carcinoma induced down-regulation of PKA and PKB/Akt leading to modulation of the Wnt and the NF-κB signal transduction pathways (22-24). Interestingly, in another study in which we looked at normal splenocytes, we observed an opposite response to A_3AR activation in which PI3K, PKB/Akt, IKK and NF-κB were up-regulated, resulting in an increased production of granulocyte colony stimulating factor (G-CSF) (25). It thus seems that in the present study the inflammatory cells responded to IB-MECA similarly to the tumor cells but in a different way from that of normal cells, suggesting that receptor density may affect the response to a given agonist. The differential response of pathological (tumor /inflammatory) cells vs. normal cells to A_3AR activation suggests the specificity of this receptor target.

Interestingly, caspase-3 was also up-regulated in the paw extracts derived from IB-MECA treated animals, supporting the notion that apoptosis took place.

The present study shows that IB-MECA belongs to the family of new drugs that in addition to their anti-inflammatory effect, are capable to combat bone loss. It was reported that Infliximab (anti-TNF-α monoclonal antibody) and Etanercept (soluble TNF-α receptor) have a significant beneficial effect on bone resorption in RA patients, via the inhibition of osteoclastogenesis (26-28). In distinction from these drugs IB-MECA modulates a cascade of molecular events that may evoke a broader spectrum of effects which control both survival and apoptosis. An additional advantage of IB-MECA is its chemical profile of a small orally bioavailable molecule. Moreover, in Phase I and Phase IIa clinical trials, the IB-MECA (designated as CF101) was found to have an excellent safety and efficacy profile (29, 30).

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