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Adenosine analogs and electromagnetic fields inhibit prostaglandin E_2 release in bovine synovial fibroblasts

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Summary

Objective: To investigate the role of adenosine analogs and electromagnetic field (EMF) stimulation on prostaglandin E_2 (PGE_2) release and cyclooxygenase-2 (COX-2) expression in bovine synovial fibroblasts (SFs).

Methods: SFs isolated from synovia were cultured in monolayer. Saturation and binding experiments were performed by using typical adenosine agonists: *N*6-cyclohexyladenosine (CHA, A_1), 2-[*p*-(2-carboxyethyl)-phenetyl-amino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680, A_{2A}), 5′-*N*-ethylcarboxamidoadenosine (NECA, non-selective), *N*6-(3-iodobenzyl)2-chloroadenosine-5′-*N*-methyluronamide (Cl-IB-MECA, A_3). SFs were treated with TNF-α (10 ng/ml) and lipopolysaccharide (LPS) (1 μg/ml) to activate inflammatory response. Adenosine analogs were added to control and TNF-α- or LPS-treated cultures both in the absence and in the presence of adenosine deaminase (ADA) which is used to deplete endogenous adenosine. Parallel cultures were exposed to EMFs (75 Hz, 1.5 mT) during the period in culture (24 h). PGE_2 release was measured by immunoassay. COX-2 expression was evaluated by RT-PCR.

Results: TNF-α and LPS stimulated PGE_2 release. All adenosine agonists, except for Cl-IB-MECA, significantly inhibited PGE_2 production. EMFs inhibited PGE_2 production in the absence of adenosine agonists and increased the effects of CHA, CGS 21680 and NECA. In ADA, the inhibition on PGE_2 release induced by CHA, CGS and NECA was stronger than in the absence of ADA and the EMF-inhibitory effect was lost. Changes in PGE_2 levels were associated to modification of COX-2 expression.

Conclusions: This study supports anti-inflammatory activities of A_1 and A_{2A} adenosine receptors and EMFs in bovine SFs. EMF activity appears mediated by an EMF-induced up-regulation of A_{2A} receptors. Biophysical and/or pharmacological modulation of adenosine pathways may play an important role to control joint inflammation.
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Key words: Adenosine receptors, Synovial fibroblasts, Electromagnetic field, PGE_2.

Introduction

Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common degenerative diseases of the joints, characterized by the progressive and permanent degradation of the articular cartilage, synovial hypertrophy and change in underlying bone. Although the pathophysiologic events of OA and RA are quite different, inflammation and altered synovial fibroblasts (SFs) activities are observed in affected joints[1–6]. In RA, SFs play a central role to the pathogenesis of joint destruction by an increased proliferation and the secretion of a wide range of pro-inflammatory mediators, including cytokines, growth factors, and lipid mediators of inflammation[3,6]. Pro-inflammatory mediators produced by SFs are detrimental to articular cartilage also in OA, although SFs play a less central role than in RA[1,2]. Furthermore, SFs are involved in cartilage destruction *via* the secretion of matrix degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases[7–9]. Prostaglandins, in particular prostaglandin E_2 (PGE_2), are important lipid inflammatory mediators produced by SFs and their levels are increased in the synovial fluid and synovial membrane of patients with joint diseases[10–13]. PGE_2 is synthesized from arachidonic acid *via* the actions of cyclooxygenase (COX) enzymes, either constitutively or in response to cell trauma, stimuli, or signaling molecules such as interleukin-1β (IL-1β) or tumor necrosis factor alpha (TNF-α) and it accounts for many of the pro-inflammatory actions induced by these peptides[14–16]. PGE_2 contributes to vasodilatation, vascular permeability, pain, cytokine and proteinase production in inflamed tissues[17–19]. Accordingly, the use of non-steroidal anti-inflammatory drugs (NSAIDs) and/or selective COX-2 inhibitors which control inflammation and inhibit PGE_2 production represent the standard recommended treatment of OA and RA[20–22]. Alternative potential therapeutical approaches to control inflammation and manage joint diseases are suggested by studies which investigated the role of adenosine[23–27] and biophysical stimulation by electromagnetic fields (EMFs) in cartilage

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and arthritic diseases[28]. Adenosine, interacting with four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) acts as a potent endogenous inhibitor of inflammatory processes in several tissues[29–32]. In SFs and chondrocytes adenosine has been involved in the production of MMPs and inflammatory mediators[23–25,33,34]. *In vivo* adenosine analogs inhibit joint destruction when used in the treatment of adjuvant induced arthritis and septic arthrosis[25,26,35]. EMFs display several effects on different tissues, including bone and cartilage. *In vitro*, EMFs increase cytokine and growth factors production[36] and stimulate osteoblasts and chondrocytes proliferation[37,38]. In cartilage explants, EMF exposure increases proteoglycan synthesis, prevents the catabolic effect of the pro-inflammatory cytokine IL-1 and acts in synergy with insulin-like growth factor-I[39–41]. Further, *in vivo* EMFs preserve the morphology of articular cartilage and retard the development of osteoarthritic lesions in guinea pigs; in humans they appear useful for the treatment of OA and to control joint inflammation after arthroscopic surgery[42–45]. Recent studies, showing that EMFs evoke a specific up-regulation of the A_{2A} and A_3 adenosine receptors, suggest that EMFs may have anti-inflammatory activities mediated by the up-regulation of adenosine receptors[46–48].

On the bases of the above observations and of the role of SFs to elicit and to maintain joint inflammation, we investigated if adenosine receptor agonists and EMF biophysical stimulation, alone or combined, might limit PGE_2 release in SFs treated with known inflammatory stimuli: TNF-α and the bacterial lipopolysaccharide (LPS)[8,11,12,49]. COX-2 expression was evaluated by RT-PCR as it appears to be the primary enzyme controlling PGE_2 synthesis in response to inflammatory stimuli[10,16]. Further, adenosine analogs were used to characterize the presence of adenosine receptors and their affinity in bovine SFs, in the absence and in the presence of EMFs.

Materials and methods

SF CULTURES

SFs were obtained by culture of the bovine synovial fluid, aspirated from the metacarpophalangeal joints of 14–18-month-old animals, as previously described[46,50]. SFs at the third-fourth passage were used in the experiments. Cells were characterized by immunofluorescence staining with vimentin, a marker for fibroblasts[51]. To confirm that SF cultures were not contaminated by macrophages, CD14 expression was evaluated by RT-PCR.

IMMUNOFLUORESCENCE STAINING

SFs were fixed with cold methanol, washed with phosphate-buffered saline (PBS) and incubated with the primary monoclonal antibody (mAb) for the human vimentin (Sigma–Aldrich, Italy) at 1:200 dilution for 1 h at 37°C. Washed slides were then incubated with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibody for 1 h at 37°C. Nuclei were stained with the DNA dye, 4′,6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml in PBS ethylene glycol tetraacetic acid (EGTA)) for 10 min. Fluorescence was visualized using the Nikon Eclipse TE 2000-E microscope (Nikon Instruments, Italy) equipped with a digital camera (DXM 1200F).

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

CD14 and COX-2 expression in SFs cultures was assayed by RT-PCR. Total RNA extraction was performed by a commercial kit (RNeasy Kit, Qiagen, Deutschland). RNA conversion to cDNA was performed by the kit SuperscriptTM First-Strand Synthesis System (Invitrogen, USA). Oligonucleotide primers for CD14 were dp5′-CTGGAAGCCGGCG-3′; rp5′AGCTGAGCAGGAACCTGTGC-3′ and oligonucleotides for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were dp5′-TGGCAT CGTGGAGG-GACTTAT-3′; rp5′-GACTTCAACAGCGACACTCAC-3′. Sequences were selected to amplify both human and bovine genome. Oligonucleotides for COX-2 were dp5′-TCCAGATCACATTTGATTGACA-3′; rp5′-TCTTTGACTGTGGGAGGATACA-3′[52]. Oligonucleotides sequences were from separate exons to exclude genomic DNA contaminations. Two microliters of cDNA were amplified by the specific oligonucleotide sets and PCR reactions were performed as previously described[46]. Cycling parameters were as follows: 1 min at 94°C; 1 min at the specific annealing temperature (55°C for CD14, 61°C for GAPDH, 60°C for COX-2); and 1 min at 72°C. PCR product sizes were 403 bp for CD14, 370 bp for GAPDH and 450 bp for COX-2. mRNA from human macrophages was used as a positive control for CD14 expression. PCR products were analyzed on 1.5% agarose gel, stained with ethidium bromide and visualized under UV.

CHARACTERISTICS OF EMFs

The EMF generator system used in binding and PGE_2 functional assays was the same used in previous studies (I-ONE, Igea, Carpi, Italy)[39–41,46–48]. The magnetic field was generated by a pair of circular coils of copper wire placed opposite to each other. The coils were powered by the generator system, which produced the input voltage of pulse. The pulse duration of the signal was 1.3 ms and the repetition rate 75 Hz, yielding a duty cycle of 1/10. The intensity peak of the magnetic field was 1.5 mT and the induced electric field, as detected with a standard coil probe (50 turns, 0.5 cm internal diameter of the coil probe, 0.2 copper diameter), was 0.07 mV/cm.

SATURATION AND COMPETITION BINDING EXPERIMENTS TO ADENOSINE RECEPTORS

SFs, used for cellular membrane preparations, were washed with PBS and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4). The cell suspension was homogenized by a Polytron, centrifuged for 30 min at 100,000*g* and used in saturation and competition binding experiments[46–48]. Saturation experiments to A_1 receptors were performed using [^{3}H]-*N*6-cyclohexyladenosine ([^{3}H]-CHA, 34.4 Ci/mmol; NEN – Perkin Elmer, USA) as radioligand for an incubation time of 90 min[53]. Non-specific binding was determined in the presence of CHA 1 μM. Saturation experiments to A_{2A} receptors were performed using [^{3}H]-2-[*p*-(2-carboxyethyl)-phenetyl-amino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]-CGS 21680, 39.6 Ci/mmol; NEN – Perkin Elmer, USA) as radioligand with an incubation time of 120 min[54]. Non-specific binding was determined with CGS 21680 1 μM. Saturation experiments to A_{2B} receptors were performed using [^{3}H]-*N*-benzo [1,3[dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yl-oxy]-acetamide ([^{3}H]-MRE 2029F20, 123 Ci/mmol; Amersham Laboratories, UK) as radioligand for 60 min[55]. Non-specific binding was determined with MRE 2029F20 1 μM. Saturation experiments to A_3 receptors were performed using [^{125}I]-4-aminobenzyl-5′-*N*-methylcarboxamidoadenosine ([^{125}I]-AB-MECA, 2000 Ci/mmol; Amersham Laboratories, UK) as radioligand, for 60 min[56]. Non-specific binding was determined with AB-MECA 1 μM. In competition experiments, carried out to determine A_1, A_{2A}, A_{2B} and A_3 affinity values, 1 nM radioligands were used on SF membranes (100 μg protein per assay) and 6–8 different concentrations of the examined agonists. At the end of incubation time, in binding experiments performed in unexposed and EMF-exposed SF membranes, bound and free radioactivities were separated by filtering the assay mixture through Whatman GF/B filters (Brandel Harvester) and radioactivity was counted (Scintillation Counter Packard Tri Carb 2500TR).

SF TREATMENTS WITH ADENOSINE AGONISTS AND EMF EXPOSURE

For the analysis of PGE_2 release, SFs at third-fourth passage were plated at 10,000/cm^2 in complete medium (Dulbecco's modified Eagle's/Ham's F12 (1:1) medium (DMEM/F12) containing 10% fetal bovine serum (FBS) and antibiotics) in multiwells (Nunc, Denmark, 1.6 cm the diameter of each well) and used after 5 days plating. In preliminary experiments increasing doses of the recombinant human TNF-α (Preprotech, USA) and the bacterial LPS (Sigma, USA) selected in the range of those used in previous studies, were tested[11,12,49]. In the following experiments TNF-α and LPS were used, respectively, at 10 ng/ml and 1 μg/ml, which elicited maximal PGE_2 increase in preliminary experiments. Control cells were incubated in complete medium alone. In a first series of experiments adenosine analogs were added to both control and TNF-α- or LPS-treated cultures in the presence of endogenous adenosine. The adenosine agonists CHA (A_1), CGS 21680 (A_{2A}), 5′-*N*-ethylcarboxamidoadenosine (NECA) (non-selective), and *N*6-(3-iodobenzyl) 2-chloroadenosine-5′-*N*-methyluronamide (Cl-IB-MECA) (A_3) were used at 1 μM (Sigma, USA). In a second series of experiments, treatments with adenosine agonists in the presence of TNF-α or LPS were performed in complete medium containing 2 IU/ml adenosine deaminase (ADA, Fluka–Sigma–Aldrich, Switzerland) to deplete endogenously released adenosine. Different ADA concentrations (0.5–4 IU/ml) were analyzed on PGE_2 release and cell viability to evaluate the effect of endogenous adenosine.

To investigate the effects of EMFs on PGE_2 production, cultures treated as described above, were exposed to EMFs during the whole treatment period.

In some experiments, 1 μM forskolin (Sigma, USA), a direct activator of adenylate cyclase, was added to both control and TNF-α-treated cultures in the absence and the presence of EMFs[57]. At each condition tested, after 24 h treatment, medium was removed from the well, stored at −80°C for subsequent determination of PGE_2 and the monolayer protein content was evaluated[58].

PGE_2 ASSAY

The concentration of PGE_2 was measured using a commercially available competitive enzyme immunoassay according to the manufacturer's instructions (PGE_2 Assay, R&D Systems, Inc., Minneapolis, USA). Samples and standards were assayed in duplicate. PGE_2 production was normalized to the total protein content and expressed as pg PGE_2/μg protein.

MTT ASSAY

Effects of ADA on cell proliferation and viability of SFs were evaluated by the MTT assay[59,60]. Briefly, 100 μl of MTT solution (5 mg/ml in PBS) (Sigma–Aldrich, UK) were added to each well and incubated at 37°C for 3 h. The medium was then discarded and 500 μl of isopropanol/HCl 0.04 N were added to each well for the formazan solubilization. The solution absorbance was measured at 540 nm (Cary-50, UV–Visible Spectrophotometer, Varian).

STATISTICAL ANALYSIS

Data were obtained from at least five independent experiments. Each experiment was performed in triplicate. All values are expressed as mean ± S.E. of independent experiments. Analysis of data was done with Student's *t* test. Differences were considered significant at a value of $P < 0.05$.

Results

PHENOTYPE CHARACTERIZATION OF SFs

SFs used in our experiments are shown in Fig. 1. Cells showed a fibroblast-like morphology and the expression of vimentin, the main intermediate filament protein in mesenchymal cells and SFs[51]. Results obtained by RT-PCR showed the absence of CD14 expression, indicating the absence of contaminating macrophages[8].

EVALUATION OF ADENOSINE RECEPTOR AFFINITY AND DENSITY BY SATURATION AND COMPETITION BINDING EXPERIMENTS

Table I reports the affinity (K_D, nM) and density (B_{max}, fmol/mg protein) of A_1, A_{2A}, A_{2B} and A_3 receptors in unexposed or EMF-exposed SF membranes. The affinity values of adenosine receptor subtypes and the B_{max} values of A_1 and A_{2B} receptors were strictly similar in both EMF-treated and untreated cells. In contrast, after EMF treatment, the B_{max} values of A_{2A} and A_3 receptors were increased of 2.33- and 2.15-fold in comparison to control, respectively, ($^*P < 0.01$ vs untreated cells, Table I). Table II reports the affinity values (K_i, nM) of CHA, NECA, CGS 21680 and Cl-IB-MECA in untreated and EMF-treated bovine SFs. The major affinity of CHA ($K_i = 3.5$ nM) was reported in [^{3}H]-CHA binding suggesting a high affinity vs A_1 receptors. NECA and CGS 21680 showed similar affinity for A_{2A} receptors even if NECA had a good affinity also for the other subtypes. Cl-IB-MECA showed a high affinity vs A_3 receptors with a K_i value of 2.3 nM. In summary results of Tables I and II showed that EMFs induced an increase in A_{2A} and A_3 receptor density (B_{max} values) without modifying the receptor capability to bind the specific ligands (K_D, K_i values or affinity). Saturation and competition binding experiments allowed to select, on the basis of the K_i values, the adenosine agonist concentrations to be used in subsequent experiments.

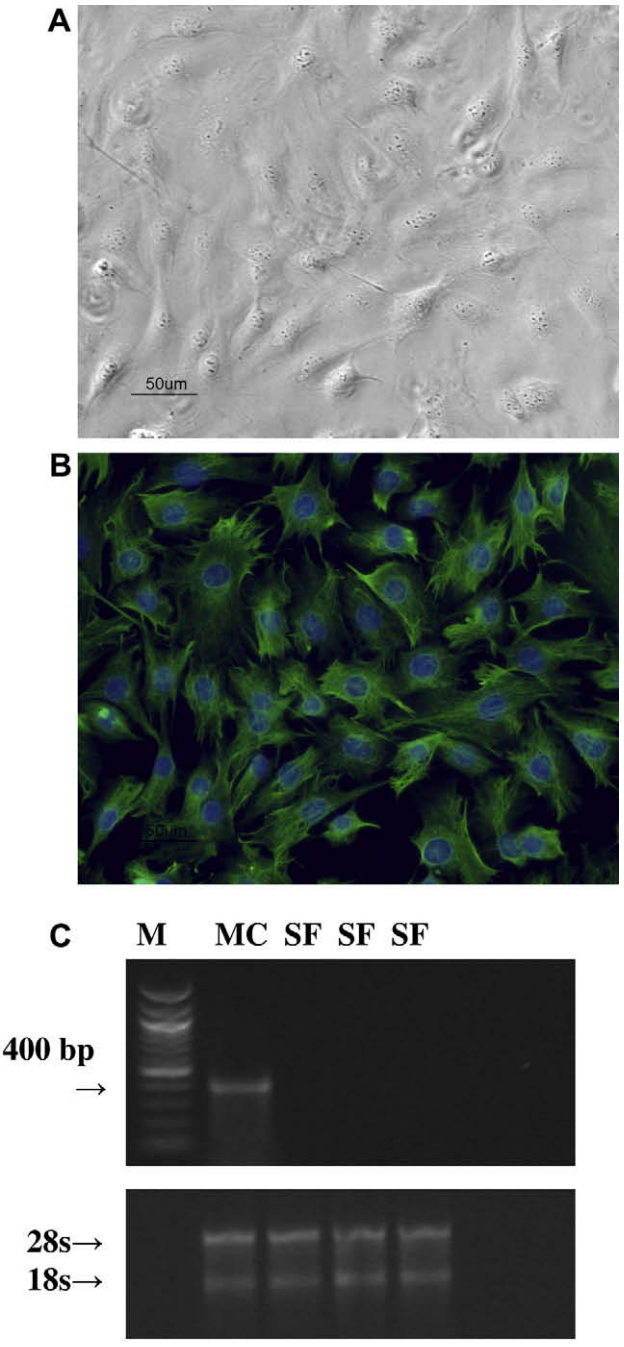

Fig. 1. Bovine SFs in culture. (A) Phase contrast. (B) Vimentin expression by immunofluorescence. Nuclei were counterstained in blue with DAPI. (C) CD14 mRNA expression in macrophages (MC) and in bovine SFs (C, upper panel). M is 100 bp DNA ladder marker (Biolabs). One microgram of total RNA was loaded for lane and stained with ethidium bromide to confirm equal RNA quantity used for RT-PCR (C, lower panel).

TNF-α AND LPS INDUCE A DOSE RESPONSE INCREASE ON PGE_2 RELEASE IN SFs

In preliminary experiments we investigated the effects of increasing doses of TNF-α and LPS, which are known to stimulate PGE_2 production in human SFs, on PGE_2 release

Table I
Affinity and density of adenosine receptors in bovine SFs

SFs	[^{3}H]-CHA binding	[^{3}H]-CGS 21680 binding	[^{3}H]-MRE 2029F20 binding	[^{125}I]-AB-MECA binding
Untreated	$K_D = 1.82 \pm 0.16$ nM $B_{max} = 24 \pm 3$ fmol/mg protein	$K_D = 4.6 \pm 0.5$ nM $B_{max} = 62 \pm 6$ fmol/mg protein	$K_D = 1.32 \pm 0.12$ nM $B_{max} = 78 \pm 8$ fmol/mg protein	$K_D = 1.65 \pm 0.15$ nM $B_{max} = 65 \pm 6$ fmol/mg protein
EMF treated	$K_D = 1.78 \pm 0.17$ nM $B_{max} = 22 \pm 3$ fmol/mg protein	$K_D = 4.9 \pm 0.4$ nM $B_{max} = 145 \pm 12$ fmol/mg protein*	$K_D = 1.26 \pm 0.11$ nM $B_{max} = 74 \pm 7$ fmol/mg protein	$K_D = 1.54 \pm 0.14$ nM $B_{max} = 140 \pm 13$ fmol/mg protein*

Data are expressed as the mean ± S.E. ($n = 4$). *$P < 0.01$ vs untreated cells.

in bovine SF cultures (Fig. 2)[11,49]. PGE_2 production by control SFs was at very low levels. TNF-α and LPS significantly increased PGE_2 production in a dose-dependent manner. The most efficient doses were 10 ng/ml for TNF-α and 1 μg/ml for LPS yielding a maximal 7.9- and 9.8-fold increase, respectively. On these results, these TNF-α and LPS doses were used to stimulate PGE_2 production in subsequent experiments.

ADENOSINE AGONISTS AND EMF EXPOSURE INHIBIT PGE_2 RELEASE IN TNF-α- OR LPS-TREATED SFs IN THE PRESENCE OF ENDOGENOUS ADENOSINE

The effects of adenosine agonists and EMFs on PGE_2 release in TNF-α or LPS unstimulated and stimulated SFs, in the presence of endogenous adenosine, are shown in Fig. 3. Treatment of cells with the A_1 agonist CHA, the A_{2A} agonist CGS 21680, the non-selective agonist NECA and the A_3 agonist Cl-IB-MECA did not modify basal PGE_2 production in the absence of TNF-α or LPS. Similarly, EMFs did not modify basal PGE_2 production, both in the absence and in the presence of the agonists [Fig. 3(A)].

In TNF-α stimulated SFs, all agonists, except for Cl-IB-MECA, significantly inhibited PGE_2 production. PGE_2 inhibition ranged from 38.6% in the presence of CHA to 54.9% in the presence of NECA [Fig. 3(B)]. Similar data were obtained in LPS stimulated SFs [Fig. 3(C)]. When TNF-α or LPS stimulated SFs were exposed to EMFs, PGE_2 production was significantly inhibited by EMFs, respectively, of 62.7% in the presence of TNF-α and 48.5% in the presence of LPS. Similar inhibitions were induced by EMFs in TNF-α or LPS stimulated SFs cultured in the presence of Cl-IB-MECA. Interestingly, in the presence of CHA, CGS 21680 and NECA, EMFs significantly increased the inhibitory activity of the agonists on TNF-α- or LPS-induced PGE_2 production [Fig. 3(B,C)]. The combined effects of adenosine agonists and EMFs reduced PGE_2 levels to those of unstimulated control cells.

Adenosine agonists were used at 1 μM, the concentration that assured a complete saturation of adenosine receptors as suggested from the affinity values (K_i, nM) of Table II.

Table II
Affinity (K_i) of typical adenosine agonists to bovine SFs

	[^{3}H]-CHA binding K_i (nM)	[^{3}H]-CGS 21680 binding K_i (nM)	[^{3}H]-MRE 2029F20 binding K_i (nM)	[^{125}I]-AB-MECA binding K_i (nM)
Untreated				
CHA	3.5 ± 0.4	812 ± 75	>1000	83 ± 7
NECA	22 ± 2	8.4 ± 0.8	156 ± 17	36 ± 4
CGS 21680	740 ± 70	7.2 ± 0.7	>1000	985 ± 90
Cl-IB-MECA	185 ± 22	643 ± 60	>1000	2.3 ± 0.2
EMF treated				
CHA	3.1 ± 0.3	805 ± 72	>1000	86 ± 8
NECA	20 ± 2	8.1 ± 0.8	163 ± 18	35 ± 3
CGS 21680	725 ± 65	7.5 ± 0.7	>1000	976 ± 86
Cl-IB-MECA	178 ± 18	637 ± 58	>1000	2.7 ± 0.3

Data are expressed as the mean ± S.E. ($n = 4$).

DEPLETION OF ENDOGENOUS ADENOSINE WITH ADA INCREASES BASAL PGE_2 RELEASE IN SFs

SFs were exposed to increasing doses of ADA (0.5–2 IU/ml) to determine the effects of depleting endogenous adenosine. PGE_2 release slightly but significantly increased in a dose-dependent manner with maximal effect at 2 IU/ml [Fig. 4(A)]. In parallel experiments, MTT assay was performed to verify that in our experimental conditions ADA did not modify SF proliferation or viability[24]. At all the doses, ADA had no effect on cell proliferation and viability [Fig. 4(B)].

DEPLETION OF ENDOGENOUS ADENOSINE WITH ADA POTENTIATES ADENOSINE AGONISTS' EFFECTS BUT LIMITS EMF-INHIBITORY EFFECTS ON PGE_2 RELEASE IN TNF-α- OR LPS-TREATED SFs

As 2 IU/ml ADA induced the maximal increase in PGE_2 production, this dose was used to deplete endogenous adenosine (Fig. 5). Both TNF-α and LPS significantly stimulated PGE_2 synthesis similarly to what observed in the absence of ADA. Also, Cl-IB-MECA did not modify PGE_2 levels in TNF-α or LPS stimulated cells. In TNF-α or LPS stimulated SFs, CHA, CGS 21680 and NECA induced a stronger inhibition on PGE_2 production than in the absence of ADA. In fact, CHA, CGS 21680 and NECA reduced PGE_2 levels to those of unstimulated control cells, and in these experimental conditions EMFs did not further decrease PGE_2 levels. Finally, when TNF-α or LPS stimulated SFs cultured in the presence of ADA were exposed to EMFs alone, the effect on PGE_2 release reduction was significantly lower than that observed in SFs cultured without ADA. This lower activity of EMFs in ADA was also observed in the presence of Cl-IB-MECA.

FORSKOLIN STIMULATES PGE_2 RELEASE IN ACTIVATED SFs

As shown in Figs. 3 and 5, CHA (A_1 agonist) and CGS 21680 (A_{2A} agonist), which inhibit and stimulate adenylate cyclase, respectively, decreased PGE_2 release in TNF-α or LPS stimulated SFs. To clarify the potential involvement of adenylate cyclase activity in the modulation of

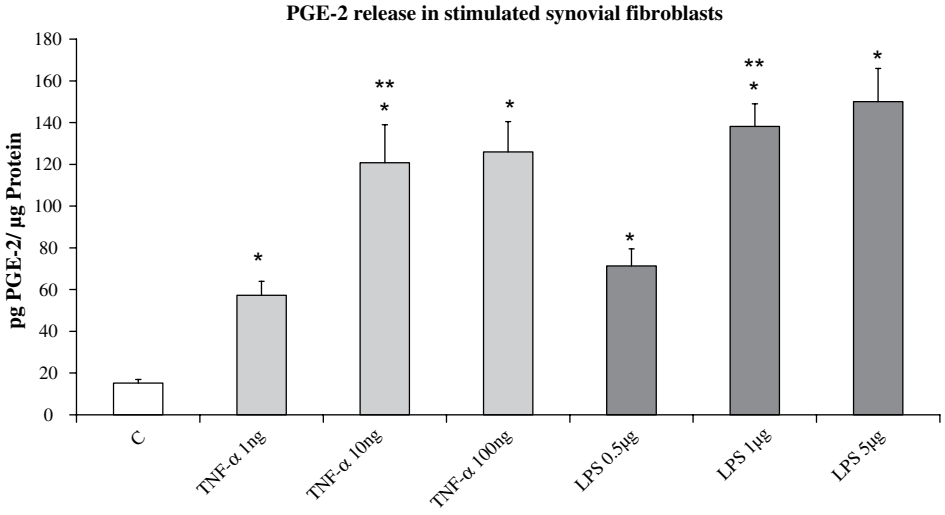

Fig. 2. Effects of increasing doses of TNF-α and LPS on PGE_2 production in bovine SFs. TNF-α and LPS induced a dose response increase on PGE_2 levels. PGE_2 levels were measured after 24 h treatment. Values are expressed as mean $\pm$ S.E. ($n = 5$). * Indicates statistical significance vs control (C). For each stimulus, ** indicates statistical significance vs the previous dose. Differences were considered significant at $P < 0.05$.

PGE_2 production, we investigated the effects of forskolin, a potent stimulator of adenylate cyclase, on PGE_2 release[57]. Forskolin did not modify basal PGE_2 levels in the absence (control = 11.8; forskolin = 14.9, pg/µg protein) and the presence of EMFs (control = 11.2; forskolin = 7.2, pg/µg protein). However, in SFs activated by the inflammatory stimuli, forskolin induced a further 24.3% increase in PGE_2 release (activated SFs = 123.1; activated SFs + forskolin = 159.3, pg/µg protein; $P < 0.05$). The effect of EMFs on PGE_2 release was not significantly modified by the presence of forskolin in culture (activated SFs + EMF = 55.4; activated SFs + forskolin + EMF = 88.2, pg/µg protein).

CHANGES IN COX-2 EXPRESSION ARE ASSOCIATED TO THE CHANGES IN PGE_2 RELEASE INDUCED BY ADENOSINE AGONISTS AND EMF EXPOSURE IN TNF-α- OR LPS-TREATED SFs

Since PGE_2 levels were regulated by adenosine agonists and EMFs, we investigated whether changes in PGE_2 release were associated to a regulation of COX-2 transcripts. COX-2 expression, evaluated by RT-PCR, at 24 h treatment, is shown in Fig. 6. As reported in literature, in our experiments the stimulation of PGE_2 synthesis induced by TNF-α and LPS was associated to an increase of COX-2 expression with respect to control cells[11,16,49]. All adenosine agonists, except for Cl-IB-MECA, inhibited COX-2 expression in TNF-α or LPS stimulated cells. EMFs inhibited COX-2 expression in both control and TNF-α or LPS stimulated SFs, also in the presence of Cl-IB-MECA. The inhibition induced by CHA, NECA and CGS on COX-2 expression in TNF-α or LPS stimulated cells was enhanced by EMF exposure, mirroring the changes observed in PGE_2 levels.

Discussion

In this study we investigated how adenosine receptor agonists and EMF exposure, alone or combined, might modify PGE_2 release in SFs treated with pro-inflammatory stimuli.

Saturation binding experiments confirmed the presence of A_1, A_{2A}, A_{2B} and A_3 receptors in SFs and that EMFs induced an up-regulation of A_{2A} and A_3 receptors, without any change in the affinity[46–48]. The affinity values calculated for each adenosine agonist, allowed to select the agonist working concentration to use in functional experiments.

In agreement with previous studies, TNF-α and LPS induced an approximately 8–10-fold increase in PGE_2 levels in SFs[11,12,49]. In a first series of experiments we analyzed the effects of adenosine agonists in the presence of endogenous adenosine. All the agonists had no effect on basal PGE_2 release, however, CHA, NECA and CGS 21680 caused a significant inhibition on PGE_2 increase induced by TNF-α and LPS. These data indicate for the first time the involvement of A_1 and A_2 receptors in the negative modulation of PGE_2 synthesis in SFs. Cl-IB-MECA did not modify PGE_2 production suggesting that the activation of the A_3 receptor is not involved in the modulation of PGE_2 synthesis. As endogenous adenosine potentially could mask the selective involvement of a specific adenosine receptor, we investigated the agonist effects also in the presence of ADA, an enzyme capable to deplete adenosine levels by its ability to convert adenosine to inosine[24]. The presence of ADA increased basal PGE_2 levels, confirming the involvement of adenosine in modulating PGE_2 production, a finding consistent with the first series of experiments and with previous studies in cartilage cells[24,27]. Further, in cells treated with TNF-α and LPS, CHA, NECA and CGS 21680 induced a stronger PGE_2 inhibition than in the absence of ADA; this can be explained by the increased potency of adenosine agonists in comparison to adenosine. Collectively, our results show that A_1 and A_{2A} adenosine receptors are involved in the inhibition of PGE_2 production in SFs. The similar effects on PGE_2 release obtained by using NECA (non-selective agonist) and CGS 21680 (A_{2A} agonist) suggest that A_{2B} receptors are not involved in this functional response. In addition, the lack of an effect of Cl-IB-MECA excludes a role for the A_3 receptors in modulating PGE_2 production.

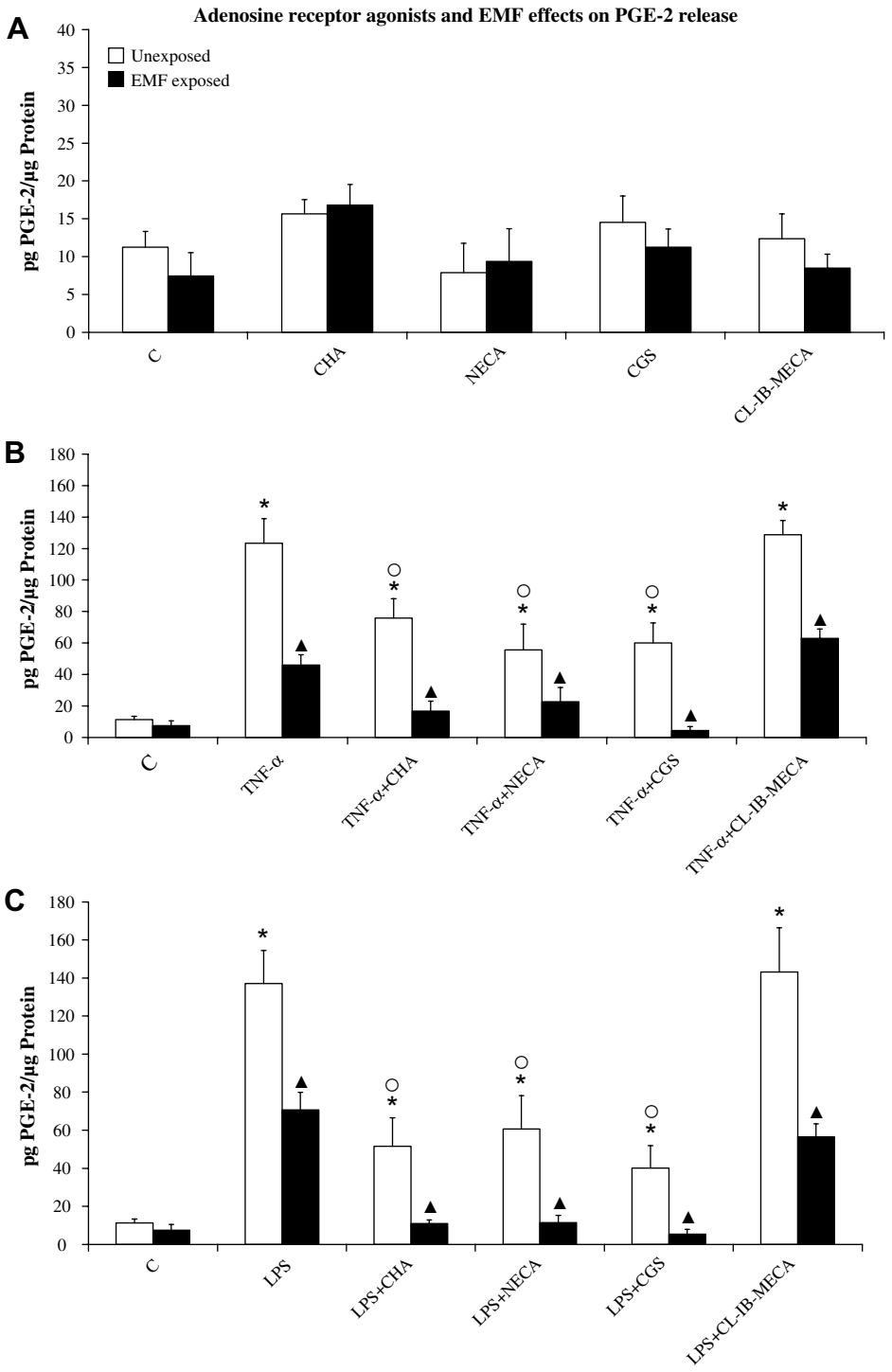

Fig. 3. Effects of adenosine receptor agonists and EMFs on basal and TNF-α- or LPS-induced PGE_2 production in bovine SFs in the presence of endogenous adenosine. Adenosine agonists and EMFs did not modify basal PGE_2 production (A) and inhibited PGE_2 release in TNF-α-treated (B) or LPS-treated (C) SFs, SF treatments in the absence of EMFs; ■ SF treatments in the presence of EMFs. Values are expressed as mean ± S.E. ($n = 6$). * Indicates statistical significance vs control (C). ▲ Indicates statistical significance vs the same treatment in the absence of EMFs. ○ Indicates statistical significance vs inflammatory stimuli (TNF-α (10 ng/ml), B; LPS (1 µg/ml), C). Differences were considered significant at $P < 0.05$.

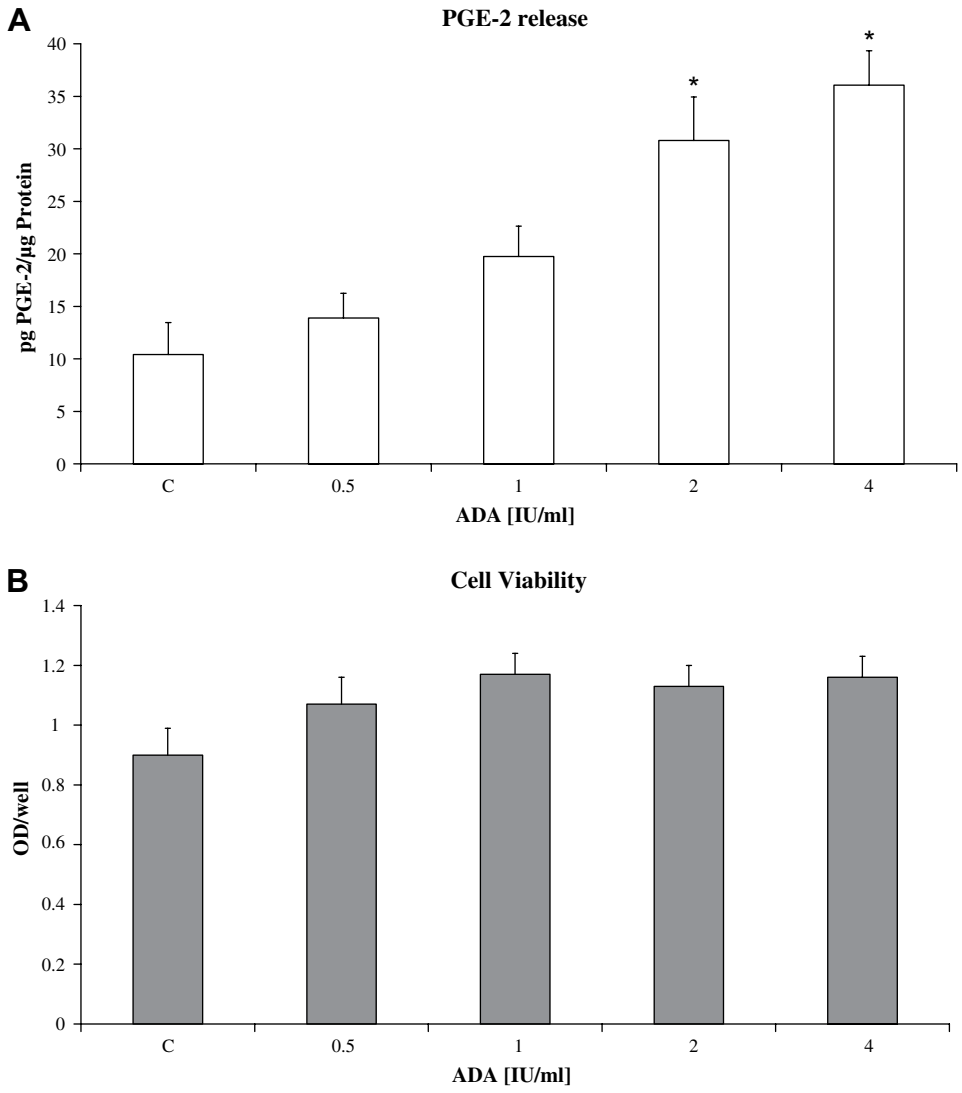

Fig. 4. Effects of depletion of endogenous adenosine with increasing doses of ADA (0.5–4 IU/ml) on PGE_2 production (A) and cell proliferation/viability evaluated by MTT test (B). Values are expressed as mean ± S.E. ($n = 5$).* Indicates statistical significance vs control (C). Differences were considered significant at $P < 0.05$.

The canonical transduction pathway coupled to A_1 and A_{2A} receptors include, respectively, the inhibition and the stimulation of adenylate cyclase with consequent reduction and increase in cAMP levels. As both A_1 and A_{2A} agonists inhibited PGE_2 release to a similar degree, this suggested that cAMP changes were not involved in the PGE_2 inhibition. Therefore, as a positive control for the adenylate cyclase activation, we investigated the effects of forskolin, a direct activator of this enzyme, on PGE_2 release[46,57]. Forskolin increased PGE_2 release in stimulated SFs confirming that the PGE_2 release inhibition observed in our experiments was not linked to cAMP production; these findings are consistent with the results described by Kojima *et al.*[61] in human SFs. Other signal transduction pathways, activated by adenosine receptors, might be involved in the negative regulation of PGE_2 production[62,63].

This and previous studies have shown that EMFs induce the up-regulation of adenosine receptors; here we evaluated the effects of EMF exposure and its possible interaction with adenosine receptor activity on PGE_2 production. Similarly to what observed for the adenosine agonists, EMFs did not modify basal PGE_2 production. However, in the presence of endogenous adenosine, EMFs strongly inhibited TNF-α- or LPS-induced PGE_2 release, respectively of 63 and 49%. Further, EMFs strongly enhanced the inhibitory activity of adenosine agonists on PGE_2 release. Since synergistic or additive actions among adenosine agonists and EMFs have not been definitely proven, our results cannot permit to drive definite conclusions concerning the EMF action mechanism. However, some lines of evidence seem to indicate that the ability of EMFs to inhibit PGE_2 production might be mediated by the adenosine pathway. In fact, the increased number of A_{2A} receptors observed in SFs exposed to EMFs (B_{max}, Table I) was associated to the enhanced inhibition of PGE_2 release induced by adenosine agonists in the presence of EMFs. Further, in conditions of depleted endogenous adenosine, the ability of EMF exposure to inhibit PGE_2

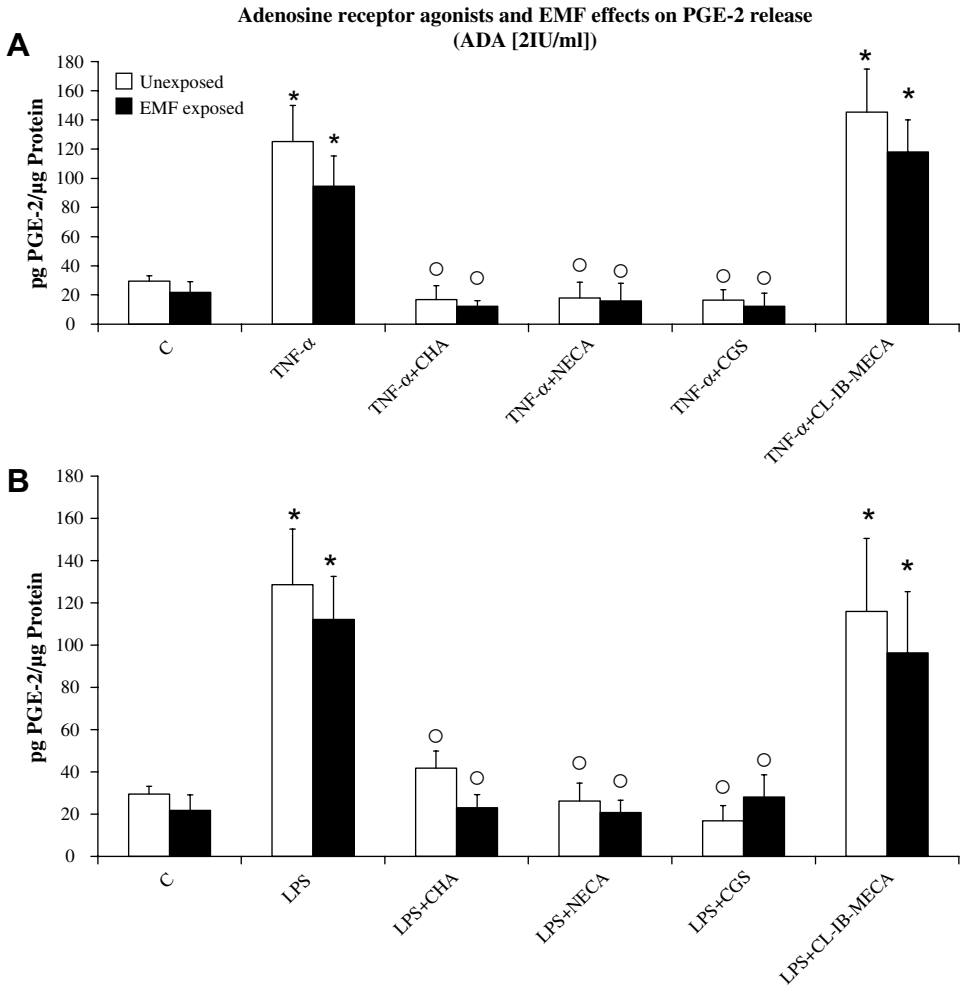

Fig. 5. Effects of adenosine receptor agonists and EMFs on TNF-α- or LPS-induced PGE_2 production in bovine SFs in the presence of ADA (2 IU/ml). Depletion of endogenous adenosine with ADA enhanced adenosine agonists effects but limited EMF-inhibitory effects on PGE_2 release in TNF-α-treated (A) or LPS-treated (B) SFs, SF treatments in the absence of EMFs; ■ SF treatments in the presence of EMFs. Values are expressed as mean ± S.E. ($n = 6$). * Indicates statistical significance vs control (C). ○ Indicates statistical significance vs inflammatory stimuli (TNF-α (10 ng/ml), A; LPS (1 µg/ml), B). Differences were considered significant at $P < 0.05$.

production in TNF-α- and LPS-treated cells was almost lost, suggesting that adenosine is necessary to mediate EMF effects. Indeed, our findings show for the first time that EMFs can inhibit inflammatory activities in SFs and act in concert with adenosine analogs by enhancing this cellular response.

Finally, our results show that the ability of adenosine agonists and EMFs to inhibit PGE_2 release is mediated by a down-regulation of TNF-α- and LPS-induced COX-2 mRNA expression. Worth of note, a similar effect is also induced in human SFs by known anti-inflammatory drugs[12].

The pharmacologic PGE_2 blockade by aspirin, NSAIDs and COX-2 inhibitors has been a useful anti-inflammatory strategy for more than a century, however, it is known that the appearance of side-effects may limit the chronic use of these drugs. The findings of the present study open new perspectives to the control of inflammation associated to joint diseases. It is to note that both adenosine and EMFs modulate chondrocyte activities too. In cartilage cells, adenosine and the A_{2A} receptor have been involved in the inhibition of inflammatory and matrix degradative events[23,24] and EMFs promote anabolic activities and prevent cartilage degradation[39–41]. Thus, previous observations and the results of this study suggest that adenosine analogs in combination with EMFs, may reduce inflammation and cartilage degradation in articular joints, by targeting both SFs and chondrocytes. Indeed, *in vivo*, the separate ability of adenosine analogs and EMFs to limit joint destruction has been previously proven in animal models[25,26,28,42,43].

In conclusion, our results add new relevant data in the analysis of adenosine anti-inflammatory activities by showing the involvement of A_1 and A_{2A} adenosine receptors in the inhibition of SFs responses to inflammatory stimuli. Further, this study shows that EMFs can regulate inflammatory parameters in SFs through the modulation of adenosine mediated anti-inflammatory pathway. From a clinical point of view, the pharmacological and/or biophysical modulation of adenosine pathways might have relevant therapeutic potential for the treatment of joint inflammatory diseases.

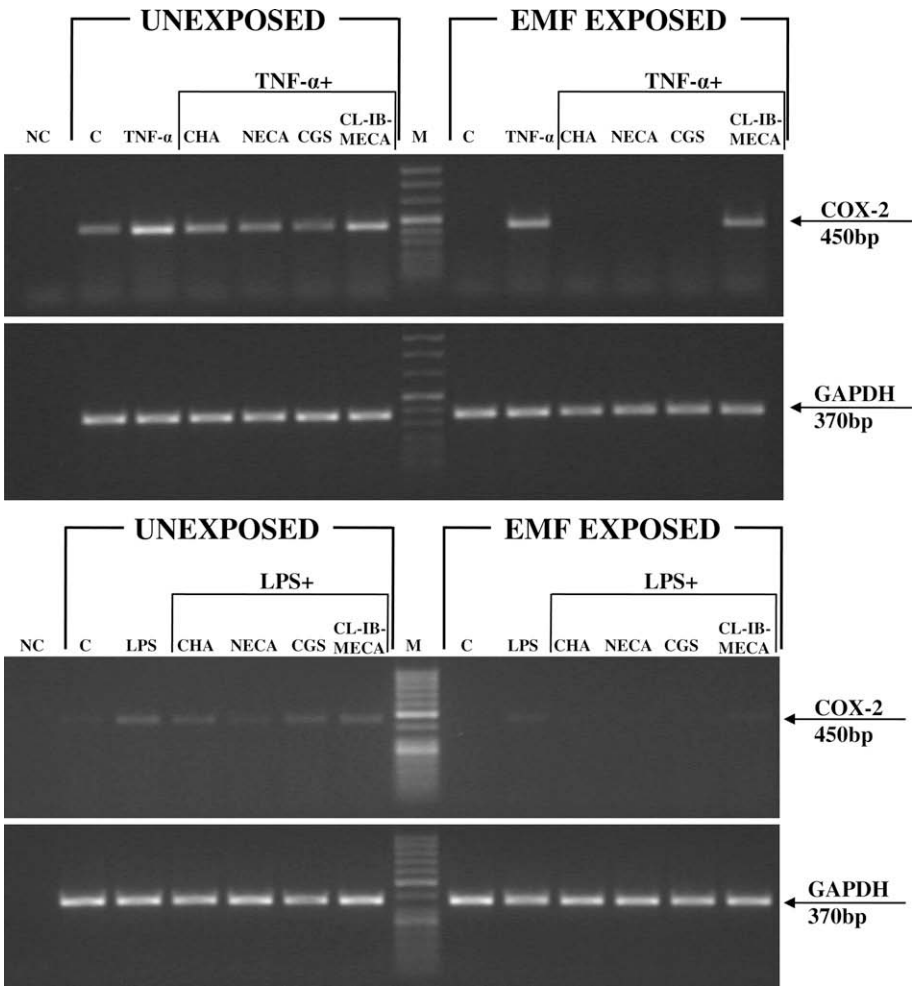

Fig. 6. Effects of adenosine receptor agonists and EMFs on control and TNF-α (upper panel) or LPS (lower panel) induced COX-2 expression evaluated by RT-PCR, at 24 h treatment. GAPDH expression was used as control gene. M is DNA molecular weight marker VIII (Roche Diagnostics GmbH, Germany).

Conflict of interest

The authors declare no conflict of interest.

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