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ORIGINAL ARTICLE

Adenosine A2A receptor activation stimulates collagen production in sclerodermic dermal fibroblasts either directly and through a cross-talk with the cannabinoid system

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Abstract Systemic sclerosis (SSc) is a connective tissue disease characterised by exaggerated collagen deposition in the skin and visceral organs. Adenosine A2A receptor stimulation (A2Ar) promotes dermal fibrosis, while the cannabinoid system modulates fibrogenesis in vitro and in animal models of SSc. Moreover, evidence in central nervous system suggests that A2A and cannabinoid (CB1) receptors may physically and functionally interact. On this basis, we investigated A2Ar expression and function in modulating collagen biosynthesis from SSc dermal fibroblasts and analysed the cross-talk with cannabinoid receptors. In sclerodermic cells, A2Ar expression (RT-PCR, Western blotting) was evaluated together with the effects of A2A agonists and/or antagonists on collagen biosynthesis (EIA, Western blotting). Putative physical and functional interactions between the A2A and cannabinoid receptors were respectively assessed by co-immuno-precipitation and co-incubating the cells with the unselective cannabinoid agonist WIN55,212-2, and the selective A2A antagonist ZM-241385. In SSc fibroblasts, (1) the A2Ar is overexpressed and its occupancy with the selective agonist CGS-21680 increases collagen production, myofibroblast transdifferentiation, and ERK-1/2 phosphorylation; (2) the A2Ar forms an heteromer with the cannabinoid CB1 receptor; and (3) unselective cannabinoid receptor stimulation with a per se ineffective dose of WIN55,212-2, results in a marked anti-fibrotic effect after A2Ar blockage. In conclusion, A2Ar stimulation induces a pro-fibrotic phenotype in SSc dermal fibroblasts, either directly, and indirectly, by activating the CB1 cannabinoid receptor. These findings increase our knowledge of the pathophysiology of sclerodermic fibrosis also further suggesting a new therapeutic approach to the disease.

Keywords Systemic sclerosis · Dermal fibroblasts · Fibrogenesis · Adenosine A2A receptor · Cannabinoid receptors

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Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterised by an exaggerated collagen synthesis and deposition, resulting in an extensive damage of the skin and several visceral organs. Although the pathogenesis of the disease is largely unknown, many data suggest that autoimmunity and vascular alterations are the main factors responsible for fibrosis development. Based on incidence and survival rates, an estimated 75,000–100,000 individuals in the USA have SSc with a higher risk in women than men (ratio ranging from 3:1 to 14:1). Two major subgroups of

the disease are recognised: (1) the *limited form*, in which fibrosis is mainly restricted to the hands, arms, and face, and (2) the *diffuse form*, a rapidly progressing disorder that affects a large area of the skin and compromises one or more internal organs leading to 45% mortality at 10 years. At the moment, no effective anti-fibrogenetic therapy is available for the disease [1].

Recent studies in cell and animal models have demonstrated the involvement of the endogenous purine adenosine in promoting the development of tissue fibrosis in different organs, via the interaction with the A2A receptor (A2Ar). More in detail, selective A2A agonists stimulate collagen production from rat and human hepatic stellate cell lines while A2A antagonists selectively counteract the development of liver fibrosis in carbon tetrachloride (CCl_4)-exposed mice [2]. Moreover, A2Ar occupancy promotes collagen synthesis from peritoneal fibroblasts and, conversely, both blockage and deficiency of the A2Ar reduce the progression of peritoneal fibrosis in a murine model [3]. Furthermore, the group of Cronstein [4, 5] have demonstrated not only the aptitude of A2A agonists to increase collagen production in primary human dermal fibroblasts, but also how A2Ar-deficient and A2Ar antagonist-treated mice were protected from developing dermal fibrosis in a bleomycin-induced murine model of scleroderma. In accordance with all these data, partially adenosine deaminase (ADA)-deficient mice, displaying high tissue adenosine levels, develop liver, renal, lung, and dermal fibrosis [6, 7]. However, no data is currently available on the expression and function of the A2Ar on human fibroblasts deriving from the skin of SSc patients.

Another current line of research documented that also cannabinoids, including natural extracts of marijuana plants as well as several synthetic derivatives, are able to modulate fibrogenesis possibly interacting with the two cannabinoid receptors currently known (named the CB1 and the CB2 [8]). The available data, suggesting an opposite effect of receptor stimulation with the CB1 promoting and the CB2 inhibiting the fibrotic process, respectively, demonstrated that the cannabinoid system is involved in liver, pancreatic and skin fibrosis [9–11]. Accordingly, in two recent investigations performed in our Institution, we demonstrated that the non-selective CB1/CB2 synthetic cannabinoid agonist WIN55,212-2 not only exerted an evident inhibiting activity on collagen biosynthesis in dermal fibroblasts from patients affected with SSc [12], but it also abrogated dermal fibrosis in vivo in a bleomycin mouse model of scleroderma [13]. These findings have been confirmed by Servettaz and coll. [14], who demonstrated that treatment with WIN55,212-2 (or with the selective CB2 agonist JWH-133) prevented the development of skin and lung fibrosis in a mouse model of diffuse SSc induced by subcutaneous hypochlorite injections.

Moreover, recent evidence in the central nervous system intriguingly suggests physical and functional interactions between the A2A and the CB1 receptors, resulting in a strict dependence for CB1 signalling on A2Ar co-activation [15]. In fact, Carriba and coll. [16] reported that CB1 and A2Ar form heteromeric complexes in co-transfected HEK-293 T cells and rat striatum where they co-immunoprecipitate and co-localise in fibrillar structures. In the same study, the authors demonstrated that in a human neuroblastoma cell line, A2Ar blockade or incubation with adenosine deaminase counteracted the ability of a CB1 agonist to inhibit forskolin-induced cAMP accumulation.

On the basis of all these considerations, we put forward the hypothesis that A2A receptor occupancy may stimulate the collagen biosynthetic process in SSc fibroblasts either directly and/or indirectly, by permitting the activation of CB1 receptor signalling. If this is the case, the concomitant use of an A2A selective antagonist may enhance the anti-fibrotic activity of WIN55,212-2 in sclerodermic cells, possibly inhibiting a parallel adenosine-dependent pathway activating fibrogenesis or, indirectly blocking the undesirable CB1 agonist effects of the molecule, or both.

The present study performed on dermal fibroblasts from SSc patients is aimed at: (1) evaluating the extent of A2Ar expression and its role in the regulation of collagen biosynthetic process; (2) confirming the formation of A2A-CB1 heteromers also in these cells; (3) testing the putative synergistic anti-fibrotic effect exerted by the concomitant modulation of the purinergic and the cannabinoid systems.

Materials and methods

Skin biopsies

Dermal fibroblasts were obtained by 5 mm punch biopsy from the leading edge of the affected skin, on the forearm of five patients affected by anti-Scl70 positive diffuse SSc and from four healthy volunteers. All subjects gave their written informed consent to the study and the protocol was approved by the local Ethical Committee. Patients were not receiving adenosine modulating substances (drugs or xanthine-containing beverages) nor taking cannabinoids for either recreational or therapeutic use at the time of biopsy. Demography of the subjects involved in the study is depicted in Table 1.

Dermal fibroblasts isolation, culture and reagents

Skin specimens were digested using 1 mg/ml clostridial collagenase (Sigma-Aldrich, Milan, Italy) in phosphate-buffered saline (PBS). Cell suspensions were plated out in 10 ml of Dulbecco's modified Eagle's medium supple-

Table 1 Demography and clinical characteristics of SSc patients and healthy controls

	SSc patients (n=5)	Healthy controls (n=4)
Sex (F/M, n)	4/1	2/2
Age (median (range), years)	49.0 (32–69)	47.5 (31–65)
Disease duration[a] (median (range), years)	5 (4–15)	–
Disease subset (limited/diffuse, n)	0/5	–
Anti-Scl-70 (positive/negative, n)	5/0	–
Treatment (n)		
Prostacyclin analogues	5	–
Endothelin-1 receptor antagonists	3	–
Calcium-channel blockers	3	–
Steroids	2	–

SSc was determined according to the Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee [17]

[a]From the first non-Raynaud's manifestation

mented with L-glutamine (2 mM), FCS (10%), penicillin (200 U/ml), and streptomycin (200 μg/ml) in 100-mm culture dishes and incubated in a humidified atmosphere containing 5% CO_2. In order to avoid changes in the original phenotype, the experiments were conducted at the third passage in fibroblast cultures from either SSc patients and healthy controls. Except where indicated otherwise, all the reagents cited above were from Euroclone (Pero, Italy).

Culture stimulation

Primary human dermal fibroblasts of sclerodermic patients and controls in early passage were grown to near confluence prior to treatment. The adenosine A2A receptor agonist CGS-21680 (1 μM) was incubated with primary human dermal fibroblasts for 24 h (37°C in 5% CO_2) with or without the A2A receptor antagonist ZM-241385 (1 μM). The putative involvement of ERK1/2 pathway in CGS-21680-induced A2A receptor activation was evaluated by adding the selective ERK1/2 inhibitor FR-180204 (50 μM) to the cultures. Experiments evaluating the putative cross-talk between adenosine and cannabinoid systems were performed by a 24-h incubation of the cultures with ZM-241385 and/or the cannabinoid receptor agonist WIN55,212-2 (0.5, 1, 10 μM).

Finally, to assess the role of CB2 receptor in such a cross-talk, we added the specific CB2 receptor antagonist AM-630 at different concentrations (ranging from 80 μM to 0.1 nM) to the cultures co-incubated with WIN55,212-2 (1 μM) and ZM-241385 (1 μM).

CGS-21680, ZM-241385, WIN55,212-2, FR-180204, and AM-630 were purchased from Tocris (Bristol, UK), and solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy). Accordingly, basal fibroblast cultures were run along with DMSO as control.

Reverse transcriptase-PCR analysis

Total RNA from fibroblasts was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) by incubation with Maloney murine leukaemia virus reverse transcriptase (200 U/μl for sample), oligo(dT) primers (0.5 μg/μl for sample) and deoxyribonucleotide triphosphate (dNTPs; 10 mM; all Invitrogen, Carlsbad, CA, USA) in 20 μl total volume. cDNA was amplified in a 50-μl reaction mixture containing Euro Taq DNA polymerase (1.25 U for sample; Euroclone, Pero, Italy), $MgCl_2$ (5 mM), dNTPs (0,25 mM), and 20 pmol of each sense and antisense primer for A2Ar: 5′-tgtcctggtcctcacgcagag-3′(forward) and 5′-cggatcctgtaggcg tagatgaagg-3′ (reverse) and for β-actin: 5′-ggatcttcatgagg tagtcagtc 3′(forward) and 5′-cctgcctttgccgatcc-3′ (reverse).

Polymerase chain reaction (PCR) was performed using a thermocycler ONE PERSONAL (Euroclone, Pero, Italy) for 35–40 cycles. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide and bands were visualised and photographed by ultraviolet transillumination.

Western blot analysis

Dermal fibroblasts were washed twice in ice-cold PBS and lysed on ice with lysis solution: 1% Triton X-100, 150 mM NaCl, 20 mM Tris pH 8 and protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). The protein concentration of the samples was determined by the Bio-Rad protein assay (Bio-Rad Quick Start, Bio-Rad, Milan, Italy). Cell lysates were boiled at 100°C for 5 min in SDS gel loading buffer and later separated on a NuPage 3–8% Tris Acetate Gel (Invitrogen, Carlsbad, CA, USA). The proteins were then electrotransferred to nitrocellulose membranes (Pierce, Rockford, USA) for 1 h and 30 min at room temperature. The filters were blocked for 1 h at room temperature in 5% milk dissolved in Tris-buffered saline Tween (TBS-T), and then incubated (overnight, 4°C) with specific antibodies: anti-adenosine A2A receptor rabbit polyclonal IgG, anti-α1 type I collagen goat polyclonal IgG, anti-alpha-smooth muscle actin (α-SMA) mouse monoclonal IgG, anti-cannabinoid CB1 receptor goat polyclonal IgG, anti-cannabinoid CB2 receptor

goat polyclonal IgG, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA), anti-phospho-ERK-1/2, and anti-ERK-1/2 (both rabbit polyclonal IgG; Cell Signalling, Danvers, MA, USA). After 3 washes of 10 min each in TBS-T, the membranes were incubated with specific horseradish peroxidase-conjugated second antibodies for 45 min at room temperature and washed twice in TBS-T. Proteins were visualised using an ECL Western Blotting kit (Amersham Biosciences, Buckinghamshire, UK). GAPDH was determined in each filter in order to normalise slight variations in protein loading.

Collagen supernatant assay

After the different treatments, the supernatants were collected and stored at −20°C. An enzyme immunoassay (EIA) kit (Takara Bio Inc., Otsu, Japan) was used to evaluate the procollagen type I carboxy-terminal peptide (PIP) supernatant levels as an expression of the collagen release. PIP level was measured as nanograms per microgram of protein.

Co-immuno-precipitation

Co-immuno-precipitation was used to study the putative A2A-CB1 and/or A2A-CB2 receptor physical interactions in dermal fibroblasts from SSc patients and controls. The experiment was conducted in two consecutive phases: (1) the immuno-precipitation of the A2A receptor protein with anti-A2A antibody, and then (2) the immunoblotting of the precipitate with anti-CB1 or anti-CB2 antibody.

Dermal fibroblasts were lysed on ice for 30 min with buffer G (50 mM Tris HCl, 1% Triton X-100, 10% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, and protease and phosphatase inhibitors) and centrifugated for 10 min at 14,000×*g* at 4°C. Protein concentration of the samples was assayed using the Bio-Rad protein assay.

In a preclearing phase, the resina was washed two times with Buffer G and centrifugated for 5 s at 10,600×*g* at 4°C. The pellet was incubated for 30 min with cell lysates under continuous shaking at 4°C and then it was centrifugated for 5 s at 10,600×*g* at 4°C.

The supernatants (lysates) obtained were incubated for 2 h at 4°C with anti-A2A receptor; then, they were added to the pellet (Protein A-AGAROSE, Roche Applied Science, Mannheim, Germany) previously washed repeatedly with Buffer G and incubated for 1 h at 4°C while being gently agitated. The supernatants (called "Void") were constituted by any unbound protein, and they were blotting with the total protein and the immunoprecipitates.

The pellet (protein A) was washed several times with buffer G, centrifugated for 5 s at 10,600×*g* at 4°C, resuspended in sample buffer 2× (SDS, 1 M pH 6,8 Tris, glycerol; B-mercaptoethanol), and then boiled for 5 min at 100°C. Western blots were performed with anti-CB1 and anti-CB2 receptor antibodies and Void was used as a control sample.

Statistical analysis

The difference in A2Ar expression between patient and control fibroblasts was estimated by the two-tailed Mann–Whitney *U* test (A2Ar/GAPDH ratio, Western blotting) and paired "*t*" test (A2Ar/actin ratio, reverse transcriptase-PCR analysis (RT-PCR)) with the data not normally and normally distributed, respectively. Statistical evaluation of the effect of the different treatments (CGS-21680, ZM-241385, WIN-55,212-2, FR-180204, and AM-630) was performed by the one-way analysis of variance for repeated measurements (RM-ANOVA) with the data normally distributed in all the cases. Then a "post hoc" test (Tukey–Kramer test for multiple comparisons) was employed to specifically compare the effects of each different treatment. In any case, p values less than 0.05 were considered significant (GraphPad-InStat, version 3.06 for Windows 2000, GraphPad, San Diego, CA, USA; Microsoft Corp., Redmond, WA).

Results

Adenosine A2A receptor expression is increased in SSc dermal fibroblasts

RT-PCR and Western blot analyses showed that the A2Ar is expressed in dermal fibroblasts from healthy controls and SSc patients (Fig. 1a, b). Moreover, Western blot findings demonstrated that A2Ar expression was increased by about 3-fold in SSc fibroblasts compared with control cells (Fig. 1a).

Adenosine A2A receptor stimulation increases collagen production by SSc dermal fibroblasts

Since adenosine A2A receptor was present and overexpressed in SSc fibroblasts, we then performed EIA and Western blot assays to evaluate the effects of A2A agonists and antagonists on collagen production by these cells.

The selective adenosine A2Ar agonist, CGS-21680, increased PIP supernatant levels in SSc fibroblast cultures. Time-course experiments showed that the effect of CGS-21680 on PIP levels is early as the difference in PIP level in CGS-treated and untreated cells becomes detectable after 6 h. It reaches significance after 12 h, and then it remains almost stable up to 24 h (data not shown).

The effect of CGS-21680 was completely abrogated by the co-incubation with the A2Ar antagonist ZM-241385 (Fig. 2a). A similar behaviour was observed for collagen type I intracellular protein amount (Fig. 2b).

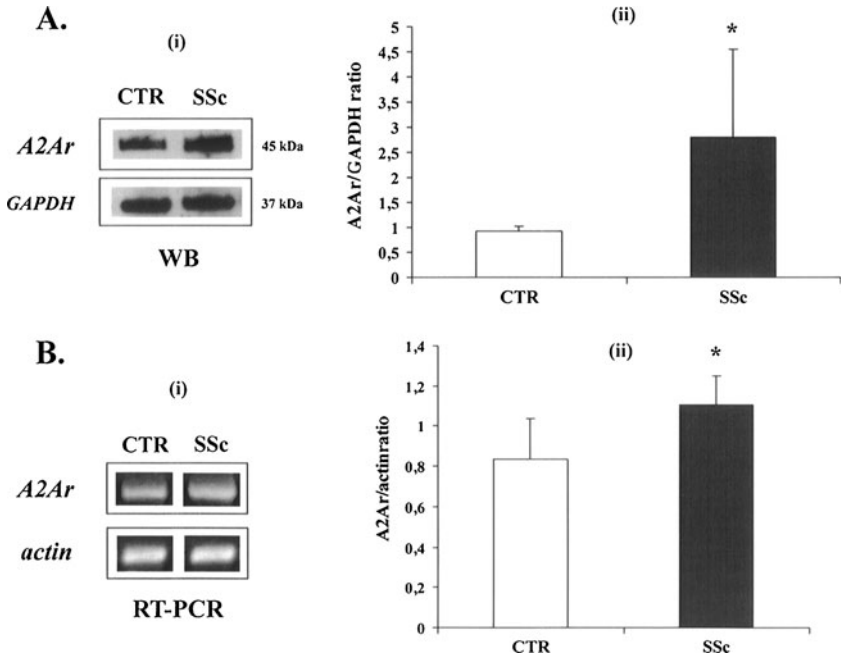

Fig. 1 Adenosine A2A receptor expression is increased in SSc dermal fibroblasts. **a** A2Ar protein expression in SSc vs. healthy dermal fibroblasts, as determined by WB analysis: (*i*) illustrative experiment and (*ii*) comparison between mean densitometric values. SSc patients, $n=5$; healthy controls, $n=4$. *$p=0.01$, two-tailed Mann–Whitney *U* test. **b** Expression of messenger RNA for A2Ar in SSc vs. healthy dermal fibroblasts, as determined by RT-PCR analysis: (*i*) illustrative experiment and (*ii*) comparison between mean densitometric values. SSc patients, $n=3$; healthy controls, $n=3$. *$p=0.01$, two-tailed paired "*t*" test. *SSc* systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *RT-PCR* reverse transcription-polymerase chain reaction, *WB* Western blotting

Specifically, EIA assay revealed that CGS-21680 was able to augment PIP supernatant level near to 40%, whereas CGS-21680+ZM-241385 co-incubation reduced the same parameter of about 50% (Fig. 2a).

On the contrary, EIA experiments performed in healthy control fibroblasts ($n=4$) did not show any significant effect of CGS-21680 and/or ZM-241385 on collagen supernatant levels (data not shown).

Adenosine A2A receptor activation induces myofibroblast trans-differentiation and ERK-1/2 phosphorylation in SSc dermal fibroblasts

In order to provide more information on the possible intracellular mechanisms involved in the A2A-mediated collagen production by SSc fibroblasts, we evaluated the effect of adenosine A2A agonists and antagonists on myofibroblast trans-differentiation and ERK-1/2 phosphorylation in these cells.

Myofibroblasts are specialised fibroblasts critically involved in the abnormal collagen production characterising SSc. The expression of the cytoskeletal protein α-SMA represents the hallmark of these cells [18]. As expected, Western blot analysis revealed that the α-SMA expression, only present in basal conditions in SSc fibroblasts, increased after A2Ar stimulation with CGS-21680 (markedly in SSc cells, slightly in healthy fibroblasts) (Fig. 3).

Increasing evidence suggests that the mitogen-activated protein kinase (MAPK) ERK-1/2, specifically the activated phosphorylated form (p-ERK-1/2), plays a key role in the SSc fibroblast activation leading to collagen production [19, 20]. Our Western blot experiments on SSc fibroblasts demonstrated the ability of the A2A agonist CGS-21680 in activating ERK-1/2 in a time-dependent manner with the maximal protein phosphorilation reached after 2–5 min (Fig. 4a). Moreover, pre-treating cells with the ERK-1/2 inhibitor FR-180204 at the concentration of 50 μM (proved to be effective in blocking CGS-21680-dependent ERK-1/2 activation; Fig. 4b) the stimulating effect of the A2A agonist on collagen production, as evaluated with PIP supernatant levels, was completely prevented (Fig. 4b).

Adenosine A2A and cannabinoid CB1 receptors co-immuno-precipitate in SSc dermal fibroblasts

Processing SSc fibroblasts for immuno-precipitation with an anti-A2Ar antibody and then analyzing the precipitate immunoblotted with an anti-CB1 antibody, we found a 54-kDa band corresponding to the molecular weight of the CB1 receptor (Fig. 5). The presence of the CB1 receptor in the immuno-precipitate obtained by using an anti-A2Ar antibody demonstrated that the two receptors co-immuno-precipitate, thus constituting an heteromer in SSc fibroblasts. The fact that similar results were also obtained in

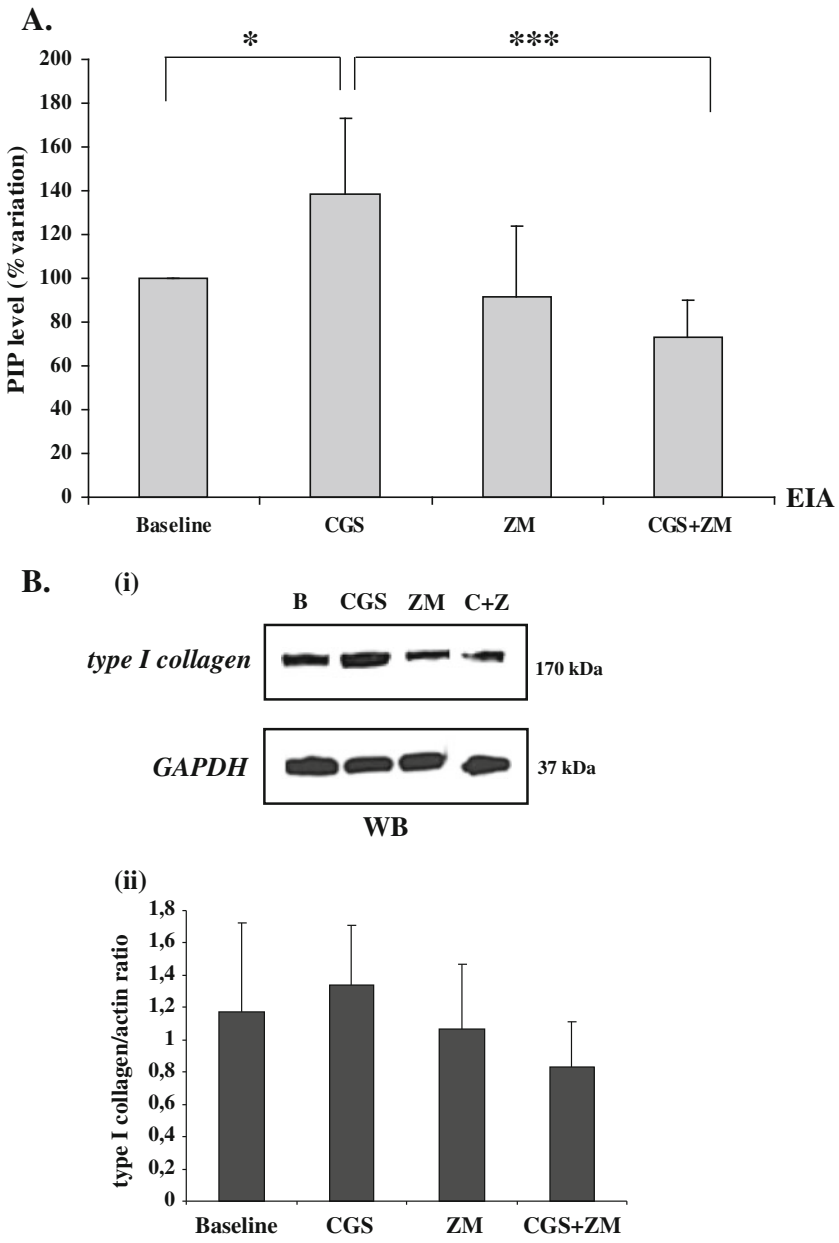

Fig. 2 Adenosine A2A receptor stimulation increases collagen production by SSc dermal fibroblasts. **a** Effect of A2Ar agonist/antagonists (CGS21680 1 μM/ZM-241385 1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline= 100%). SSc patients, $n=5$; RM-ANOVA—$p=0.001$; *$p<0.05$; ***$p<0.001$, post hoc Tukey–Kramer multiple comparison test. **b** Effect of A2Ar agonist/antagonists on collagen type I intracellular protein amount in SSc dermal fibroblasts, as determined by WB analysis: (1) illustrative experiment and (2) comparison between mean densitometric values. SSc patients, $n=2$. *SSc* systemic sclerosis, *A2Ar* adenosine A2A receptor, *PIP* procollagen type I carboxy-terminal peptide, *WB* Western blotting

healthy control cells suggests that the phenomenon does not represent a peculiarity of SSc patients but, it is a general characteristic of dermal fibroblasts.

Conversely, we found that in the same cells A2Ar and CB2 receptor do not co-immuno-precipitate thus providing evidence that the A2Ar specifically interacts with the CB1 receptor only (data not shown).

Concomitant adenosine A2A and cannabinoid receptors modulation synergistically reduces collagen production by SSc dermal fibroblasts

Starting from the above evidence that not only CB1/CB2 but also A2Ar are actively involved in SSc fibrogenesis, and that A2A and CB1 are physically associated in SSc fibroblasts, we explored the functional cross-talk putatively existing between these two systems in regulating collagen production by SSc cells.

Co-incubation of SSc fibroblasts with the non-selective CB1/CB2 synthetic cannabinoid agonist WIN55,212-2 and the selective A2Ar antagonist ZM-241385 exerted different effects on collagen production on the basis of the WIN55,212-2 concentration employed. In fact, on one hand WIN55,212-2 10 μM induced a marked reduction in PIP supernatant levels (−79%) which was not enhanced any further by the addition of ZM-241385 (Fig. 6a). On the other hand, despite the ineffectiveness of both WIN55,212-2 (0.5–1 μM) and ZM-241385 in modulating collagen production individually, co-

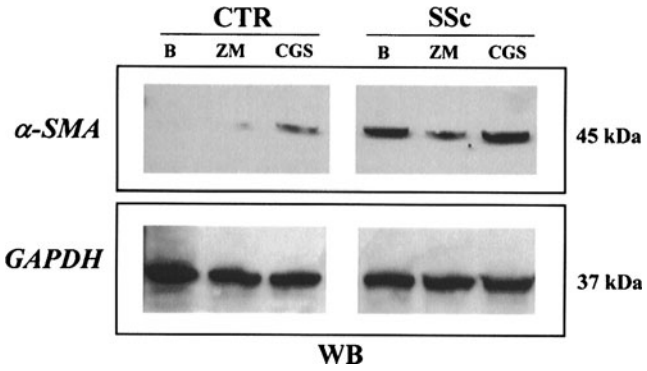

Fig. 3 Adenosine A2A receptor activation induces myofibroblast trans-differentiation in SSc dermal fibroblasts. Effect of A2Ar agonist/antagonists (CGS-21680 1 μM/ZM-241385 1 μM) on α-SMA expression in SSc and healthy dermal fibroblasts, as determined by WB analysis. *SSc* systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *α-SMA* α-smooth muscle actin, *WB* Western blotting

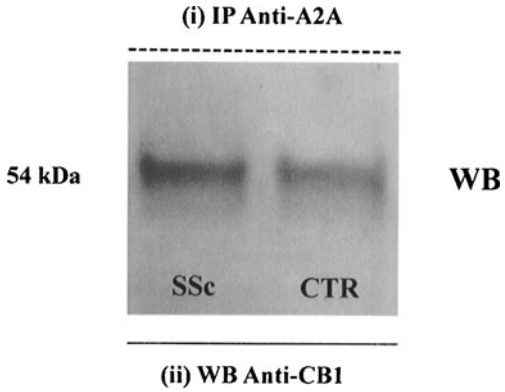

Fig. 5 Adenosine A2A and cannabinoid CB1 receptors co-immunoprecipitate in SSc and healthy dermal fibroblasts. (*i*) Dermal fibroblasts were processed for immuno-precipitation with the anti-A2A receptor antibody; (*ii*) immunoprecipitates were analysed by SDS-PAGE and immunoblotted with the anti-CB1 antibody. The band of 54 kDa indicates the position corresponding to the CB1 receptor. The experiment has been repeated three times. *SSc* systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *IP* immuno-precipitation, *CB1* cannabinoid CB1 receptor, *WB* Western blotting

incubation of 1 μM WIN55,212-2 and ZM-241385 resulted in a clear-cut inhibitory effect (−66%) similar to that observed with a high WIN55,212-2 concentration (Fig. 5b). Conversely, although co-incubating ZM-241385 with a lower WIN55,212-2 concentration (0.5 μM) led to an apparent decrease in the collagen production from the cells, such a change did not reach statistical significance (Fig. 6b).

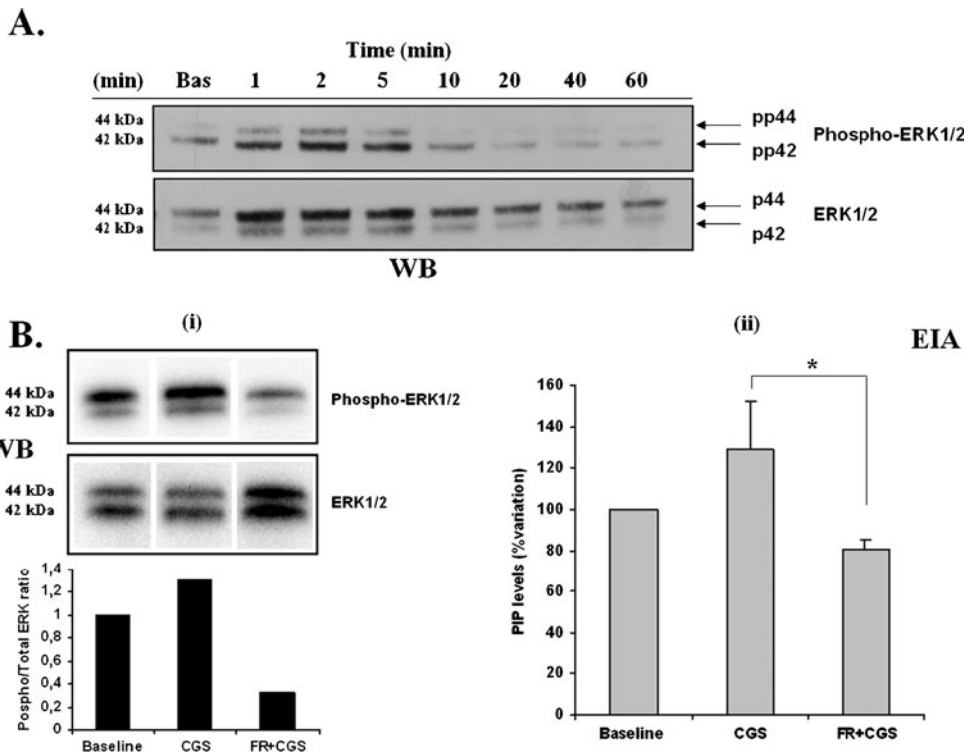

Fig. 4 Adenosine A2A receptor activation induces ERK-1/2 phosphorylation in SSc dermal fibroblasts. **a** Time course of A2Ar agonist-induced ERK-1/2 phosphorylation in SSc dermal fibroblasts, as determined by WB analysis. Serum-starved fibroblasts with 1 μM CGS-21680 for the indicated periods of time. **b** Effect of ERK-1/2 inhibitor FR-180204 (50 μM) on: (*i*) CGS-21680-induced ERK-1/2 phosphorylation, as determined by WB analysis and (*ii*) CGS-21680-induced PIP supernatant levels, as determined by EIA assay, in SSc dermal fibroblasts. Serum-starved fibroblasts with 1 μM CGS-21680 for 5 min. SSc patients, $n=3$; RM-ANOVA: $p=0.03$; *$p<0.05$, post hoc Tukey–Kramer multiple comparison test. *SSc* systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *PIP* procollagen type I carboxy-terminal peptide, *WB* Western blotting

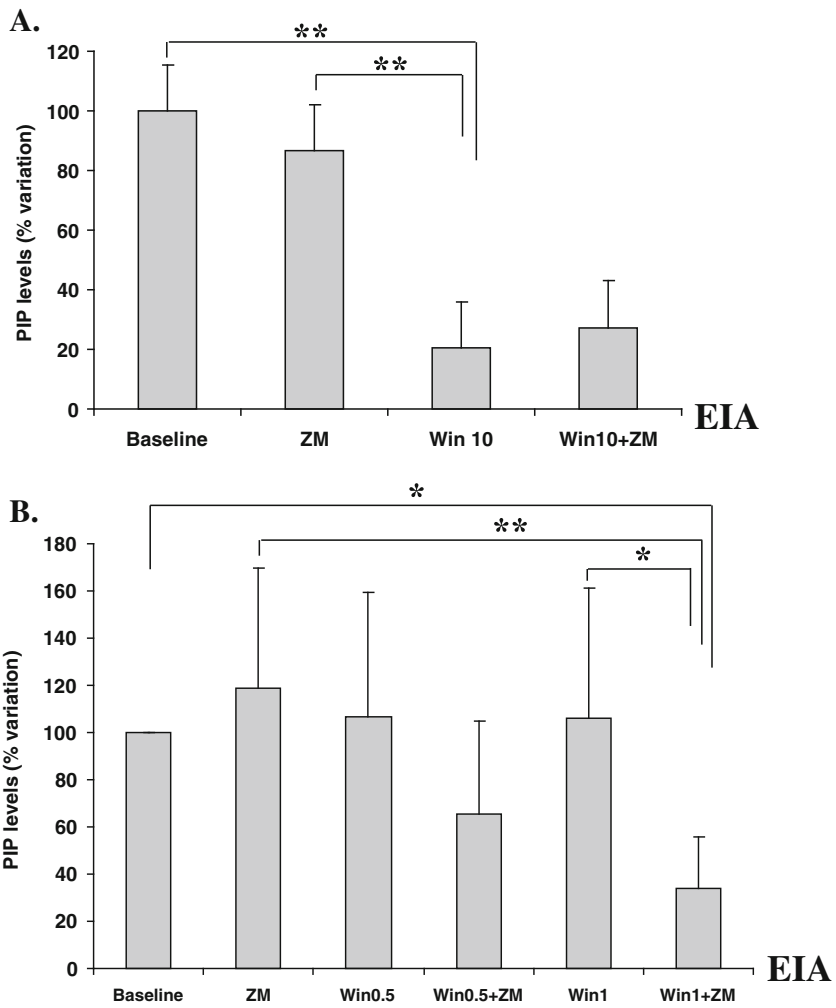

Fig. 6 Concomitant adenosine A2A and cannabinoid receptor modulation synergistically reduces collagen production by SSc dermal fibroblasts. **a** Effect of non-selective CB1/CB2 cannabinoid agonist WIN55,212-2 (10 μM) and/or A2Ar antagonist ZM-241385 (1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, $n=5$; RM-ANOVA: $p<0.001$; $^{**}p<0.01$, post hoc Tukey–Kramer multiple comparison test. **b** Effect of non-selective CB1/CB2 cannabinoid agonist WIN55,212-2 (0.5-1 μM) and/or A2Ar antagonist ZM-241385 (1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, $n=5$; RM-ANOVA: $p=0.003$; $^{*}p<0.05$; $^{**}p<0.01$, post hoc Tukey–Kramer multiple comparison test. *SSc* systemic sclerosis, *A2Ar* adenosine A2A receptor, *EIA* enzyme immunoassay, *PIP* procollagen type I carboxy-terminal peptide

Cannabinoid CB2 receptor selective activation is the main mechanism mediating the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production by SSc dermal fibroblasts

The synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production is likely the final result of a net cannabinoid CB2-stimulating effect. In fact, in these conditions WIN55,212-2 can act on the cannabinoid CB2 receptor only since the CB1 receptor is indirectly blocked by ZM-241385. To address this issue, we evaluated whether the specific CB2 antagonist AM-630 was able to remove such an inhibitory effect.

AM-630 was originally referred to as CB2 cannabinoid receptor antagonist but, it has now become clear that this compound causes activation of the CB2 receptor when employed at high concentrations (particularly in the absence of agonist binding) with opposite effects on downstream signalling cascades as those of the agonists. Accordingly, AM-630 is now referred to as cannabinoid receptor antagonist/inverse agonist [21]. On this basis, we performed a preliminary dose-finding study by adding AM-630 at different concentrations (ranging from 80 μM to 0.1 nM) to the cultures co-incubated with WIN55,212-2 and ZM-241385. As expected, we obtained a sigmoid curve in which the inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production was: (1) enhanced at higher AM-630 concentrations (80–20 μM), likely as a result of the dominant CB2 agonist-like effect of the molecule; (2) unaffected at the intermediate concentrations (10 μM–5 nM), as the CB2 agonist like and antagonist activities were substantially equivalent; (3) reduced at lower concentrations (1–0.1 nM) as a result of a dominant CB2-antagonist effect of the molecule (Fig. 7a). In particular, in cultures treated with the AM-630 concentration of 1 nM as from dose-finding experiments, collagen production was significantly increased with respect to AM-630-untreated cultures with an almost complete restoration of baseline values (Fig. 7b).

Discussion

The main findings of the present study are the following: (1) the adenosine A2Ar is overexpressed in SSc fibroblasts

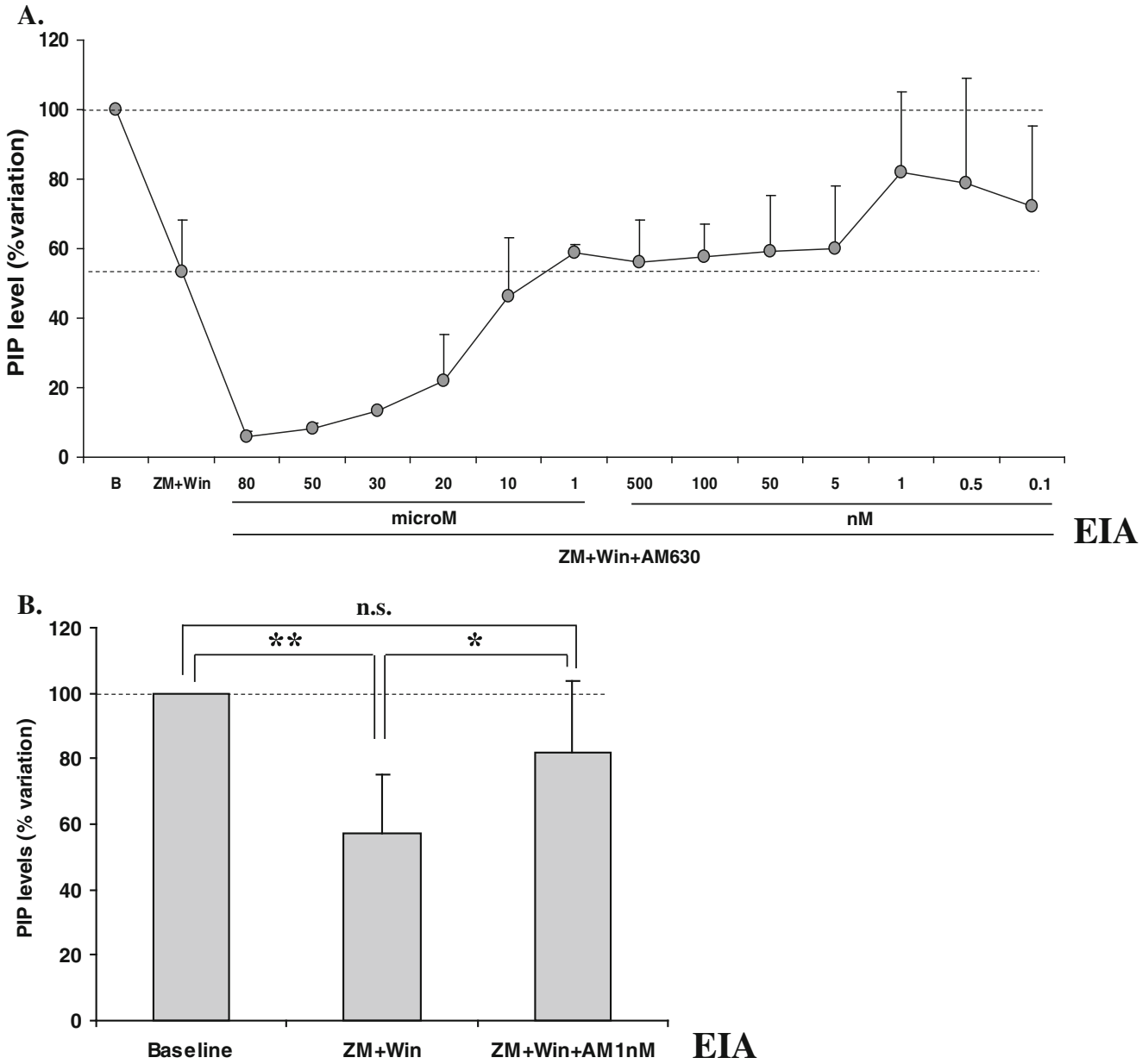

Fig. 7 Cannabinoid CB2 receptor selective activation is the main mechanism mediating the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production by SSc dermal fibroblasts. **a** Dose-finding for maximal CB2-antagonist activity of AM-630: effect on PIP supernatant levels of different concentrations of AM-630 (from 80 μM to 0.1 nM) added to SSc dermal fibroblasts in culture co-incubated with WIN55,212-2 and ZM-241385, as determined by EIA assay. **b** Effect on PIP supernatant levels of 1 nM AM-630 addition to SSc dermal fibroblasts in culture co-incubated with WIN55,212-2 and ZM-241385, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, $n=5$; RM-ANOVA: $p=0.001$; $*p<0.05$; $**p<0.01$, post hoc Tukey–Kramer multiple comparison test. *SSc* systemic sclerosis, *EIA* enzyme immunoassay, *PIP* procollagen type I carboxy-terminal peptide

and its occupancy increases collagen production, α-SMA expression and ERK-1/2 phosphorylation; moreover, in these cells (2) the A2Ar forms an heteromer with the cannabinoid CB1 receptor, and (3) following A2Ar blockade, the non-selective cannabinoid receptor stimulation with a per se ineffective dose of WIN55,212-2 results in a marked anti-fibrotic effect. Such results, considered as a whole, suggest that in SSc fibroblasts A2Ar activation may exert a pro-fibrotic activity either directly, and indirectly, via a cross-talk with the cannabinoid receptor system.

Recent evidence suggests that adenosine A2Ar plays a key role in the development of dermal fibrosis. Chan et al. [4] demonstrated that in primary human dermal fibroblasts A2Ar stimulation promoted collagen production and, this

process, in part working via the MEK-1/2/ERK pathway activation, was selectively blocked by A2A receptor antagonism. Moreover, in ADA-deficient mice, the high tissue adenosine levels deriving from the genetic defect are associated with elevated concentrations of the pro-fibrotic mediators TGF-β, CTGF, IL-6, IL-13, and PDGF in the skin as well as the development of dermal fibrosis. Both these phenomena were prevented by the pharmacological treatment with the A2A antagonist ZM-241385 [7]. Accordingly, in a murine model of scleroderma, ZM-241385 administration as well as A2Ar deletion were able to markedly attenuate bleomycin-induced dermal fibrosis as demonstrated by the reduced skin thickness, collagen content and fibrocyte accumulation [5].

These findings are strengthened and expanded by the results of the present study which confirms that the A2A-mediated activation of the fibrotic process is also relevant in human fibroblasts deriving from patients affected with SSc. In fact, in these cells, A2Ar agonism resulted in increased collagen production, α-SMA expression and ERK-1/2 phosphorilation, thereby suggesting that A2Ar stimulation may favour fibroblast trans-differentiation towards a fibrogenic phenotype (myofibroblasts) by activating the MAPKs pathway. Moreover, the fact that A2Ar agonist/antagonists employment did not result in an evident modulation of collagen production in fibroblast cultures from healthy controls suggests that in SSc cells the adenosinergic system has a particular relevance. In accordance with such considerations, we have provided evidence for the first time that the A2Ar is overexpressed in SSc with respect to control fibroblasts, thereby possibly explaining the different agonist/antagonists response that we have found. This latter finding is intriguing and, although the possibility that it represents a primitive abnormality of SSc fibroblasts cannot be ruled out, it may be likely interpreted as an adaptative response to the chronic immuno-inflammatory activation characterising the disease. In fact, a large body of evidence indicates that adenosine, by stimulating A2Ar exerts relevant anti-inflammatory and immuno-modulating activities including inhibition of pro-inflammatory cytokine release from macrophages, decrease of adhesion molecules on neutrophils and induction of an anti-inflammatory dendritic cell phenotype driving T cell responses towards a T_H2 profile [22]. It has been demonstrated that pro-inflammatory cytokines, particularly TNF-α, are able to up-regulate the A2Ar in different cell types (endothelial cells, peripheral blood mononuclear cells, neutrophils, and lung epithelial cells) [23–26] as an expression of a possible self-regulatory mechanism. Accordingly, patients with rheumatoid arthritis show an A2Ar up-regulation in lymphocytes and neutrophils (about 2–3-fold) which is normalised by the treatment with anti-TNFαdrugs [27]. On this basis, it is conceivable that also the A2Ar overexpression observed in SSc fibroblasts may be the consequence of a compensatory mechanism primarily directed to dumping immuno-inflammatory activation. However, being the receptor also able to enhance collagen production, A2Ar up-regulation may be actually maladaptative for SSc dermal fibroblasts, thereby further promoting the imbalance of these cells towards a pro-fibrotic phenotype.

The other relevant finding arising from our research is that in SSc fibroblasts a physical and functional relationship exists between adenosine A2A and the cannabinoid CB1 receptor, resulting in a cross-talk possibly involved in a critical manner in the regulation of collagen production by these cells. The evidence here provided that the A2A and the CB1 receptors co-immuno-precipitate in SSc fibroblasts (but also in fibroblasts from healthy controls) is in agreement with previous studies demonstrating that these receptors form an heteromer in the central nervous system [15, 16]. In the same studies, the authors found that CB1 receptor function is completely dependent on A2Ar co-activation: more particularly, A2Ar activation is a necessary condition for CB1 receptor signalling may occur. Our data seem to indicate that such a functional receptor relationship also operates in sclerodermic cells. In fact, we demonstrated how a relatively low concentration (1 μM) of the unselective cannabinoid CB1/CB2 agonist WIN55,212-2, per se ineffective in influencing collagen production from SSc fibroblasts, acquired a strong inhibitory effect after cell exposure to the A2A receptor antagonist ZM-241385 (also ineffective when used alone). It is conceivable that, in this condition, A2Ar blockage renders the CB1 receptor unresponsive to the agonistic effect of WIN55,212-2, thus leaving the molecule free to act on the CB2 receptor only, with the net result of a marked anti-fibrotic effect. Such a hypothesis was indeed confirmed by the fact that the CB2 receptor selective antagonist AM-630 was effective in removing the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production by SSc dermal fibroblasts.

It should be noted that in the present study we also show how the collagen production inhibiting activity of WIN55,212-2 at a concentration as high as 10 μM is not enhanced by ZM-241385, as a possible expression of a sub-maximal effect achieved at that concentration of WIN55,212-2 with no chance for any further effect. In this view, it may be also conceivable that a WIN55,212-2 concentration as low as 1 μM could be unable to produce significant receptor stimulation when the agonist is employed alone. However, our results suggest an alternative interpretation of the phenomenon. In fact, the lack of a detectable anti-fibrotic effect of 1 μM WIN55,212-2 would not be related to an insufficient CB1/CB2 receptor occupancy, but to a balanced stimulation of two receptors

(CB1 and CB2) exerting opposite, mutually neutralising effects on collagen production. In this context, the indirect CB1 blockade by the A2Ar antagonist ZM-241385 could allow the appearance of a relevant, previously masked anti-fibrotic effect of the molecule. Although WIN55,212-2 is a non-selective CB1/CB2 agonist, it has a slightly greater affinity for the CB2 rather than the CB1 receptor [28]. Thus, it is possible that a CB2 stimulation-mediated effect may arise in a significant manner for higher concentrations of WIN55,212-2 whereas it is negligible at a concentration of the agonist as low as 1 μM. If this is the case, the clear-cut anti-fibrotic effect of WIN55,212-2 at a concentration as high as 10 μM would have a clear explanation.

In conclusion, our data suggest that adenosine A2Ar occupancy is able to induce a pro-fibrotic phenotype in sclerodermic dermal fibroblasts either directly, and indirectly, by permitting CB1 cannabinoid receptor activation. These findings increase our knowledge on the pathophysiology of sclerodermic fibrosis also further substantiating the hypothesis that drugs specifically blocking the A2Ar may be useful in the treatment of SSc. Moreover, the evidence of a synergistic anti-fibrotic effect of the concomitant modulation of the adenosinergic and the cannabinoid systems in sclerodermic dermal fibroblasts suggests that a multi-target therapeutic approach to SSc may be of particular efficacy and possibly loaded with less side effects in the view of a dose-sparing effect.

Conflict of interests The authors declare no conflict of interests related to this study.

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