A2A Receptor-induced Overexpression of Pannexin-1 Hemichannels Indirectly Mediates Adenosine Fibrogenic Actions in Subcutaneous Human Fibroblasts by Favoring ATP Release

Carina Herman-de-Sousa
Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal

Maria Adelina Costa
Departamento de Química, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal

Rafaela Pedro Silva
Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal

Fátima Ferreirinha
Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal

Severino Ribeiro
Serviço de Urologia, Centro Hospitalar Universitário do Porto, 4099-001, Portugal

Paulo Correia-de-Sá (farmacol@icbas.up.pt)
Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal

---

Research Article

Keywords: Hemichannels, Fibrogenic, myofascial pain

Posted Date: August 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-787071/v1

A_{2A} receptor-induced overexpression of pannexin-1 hemichannels indirectly mediates adenosine fibrogenic actions in subcutaneous human fibroblasts by favoring ATP release

Carina Herman-de-Sousa[1], Maria Adelina Costa[1,2], Rafaela Pedro Silva[1], Fátima Ferreirinha[1], Severino Ribeiro[3], and Paulo Correia-de-Sá[1*]

[1]Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas Abel Salazar - Universidade do Porto (ICBAS-UP), Porto, 4050-313, Portugal
[2] Departamento de Química, ICBAS-UP, Porto, 4050-313, Portugal
[3] Serviço de Urologia, Centro Hospitalar Universitário do Porto, 4099-001, Portugal.

[*]Corresponding author: Paulo Correia-de-Sá, MD, PhD, Laboratório de Farmacologia e Neurobiologia / MedInUP, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) – Universidade do Porto (UP), R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Tel.: 351-220428212; fax: 351-220428090. E-mail address: farmacol@icbas.up.pt.

Abstract

Disorganization of the subcutaneous tissue due to inflammation and fibrosis is a common feature in patients with myofascial pain. Dermal accumulation of adenosine favours collagen production by human subcutaneous fibroblasts (HSCF) via A_{2A} receptors ($A_{2A}R$) activation. Adenosine mimics the fibrogenic effect of inflammatory mediators (e.g. histamine, bradykinin), which act by promoting ATP release from HSCF via pannexin-1 (Panx1) and/or connexin-43 (Cx43) hemichannels. However, this mechanism was never implicated in the $A_{2A}R$-mediated actions. NECA and CGS21680C, two enzymatically-stable $A_{2A}R$ agonists, increased Panx-1, but reduced Cx43, immunoreactivity in cultured HSCF. This effect was accompanied by increases in ATP release and collagen production by HSCF. Involvement of $A_{2A}R$ was verified upon blockage of NECA and CGS21680 effects with the selective $A_{2A}R$ antagonist, SCH442416. Inhibition of Panx1 hemichannels with probenecid also decreased ATP release and collagen production by HSCF under similar conditions. Superfluous ATP release by HSCF exposed to $A_{2A}R$ agonists overexpressing Panx1 hemichannels contributes to keep high $[Ca^{2+}]_i$ levels in the presence of inflammatory mediators, like histamine. Adenosine $A_{2A}R$-induced Panx1 overexpression was shown here for the first time; this feature indirectly implicates ATP release in the fibrogenic vicious cycle putatively operated by the nucleoside in subcutaneous tissue fibrosis and myofascial inflammatory conditions.

Introduction

The fascia is considered our largest sensory organ; it is densely innervated and plays relevant roles in scarring, tissue remodelling, inflammation, and pain perception [1-3]. Fibroblasts are the most abundant cells in fascia where they form a functional syncytium. Gap junctional (GJs) communication is preferential among adjacent fibroblasts allowing direct passage of second messengers (e.g. cyclic AMP, IP_3), metabolites, ions, and electrical signals. Fibroblasts may also interplay with each other, as well as with neuronal and inflammatory cells in the vicinity, by releasing danger molecules, like ATP, in response to noxious mechanical, inflammatory and/or neuroendocrine stimuli [4-7].

ATP-releasing hemichannels containing connexin-43 (Cx43) or pannexin-1 (Panx1) afford tuned communication between the cytosol and the extracellular environment of functionally active fibroblasts [8] , but only those channels containing Cx43 integrate the macromolecular protein scaffold of GJ communication pores [9]. Differences also exist among the two hemichannel pores concerning their regulation by phosphorylation, plasma membrane potential and extracellular calcium and magnesium concentrations; for instance, unlike Panx1-containing hemichannels, those including Cx43 are normally silent at physiological Ca^{2+} concentrations (e.g. 1.8 mM), thus requiring low external Ca^{2+} conditions to become operative [10]. Apart from the Cx43 role in intercellular communication, the carboxy tail of this protein undertakes other biological functions, such as gene transcriptional regulation and crosstalk with multiple other proteins (e.g. ZO-1, caveolin-1, c-Src, β-catenin, tubulin) [11], which have been clinically implicated in wound scar mitigation by decreasing fibroblasts movement and improving collagen alignment [12].

The cellular amounts of Cx43- and Panx1-containing hemichannels undergo substantial modifications under pathological conditions, which might have significant impact in intercellular communication [9,13-17]. Downregulation of Cx43 has been associated with improvements in skin wound healing [13,15], while its overexpression is implicated in chronic pain [14,18] and delayed healing subsequent to inflammatory conditions [19] by favouring the efflux of high ATP amounts to the extracellular microenvironment. Regarding Panx1, it integrates the inflammasome activation and mediates secretion of pro-inflammatory mediators, like interleukin-1β and histamine [5,20]. Panx1 also plays a role in the development and maintenance of chronic mechanical hypersensitivity [21], as well as in dermal fibrosis [22], most probably by releasing adenine nucleotides in response to inflammatory signal mediators [5,6].

While previous studies agree that purines are important to fascial inflammation, nociception, and connective tissue remodelling through the activation of adenine nucleotides-sensitive P2 purinoceptor subtypes, namely metabotropic $P2Y_1$, $P2Y_2$, $P2Y_{12}$ and ionotropic P2X7 [5,6,23,24], very little attention has been given to the effects of their metabolite, adenosine, acting via P1 receptors, reviewed in [25 26]. Pioneering data from our group indicates that adenosine originated from ATP breakdown tends to accumulate in the extracellular milieu of human subcutaneous fibroblast (HSCF) cultures because these cells lack extracellular adenosine deaminase (ADA) activity [5,6,27]. The close proximity between the adenosine forming enzyme, ecto-5'-nucleotidase/CD73, and the A_{2A} receptor ($A_{2A}R$) subtype favours collagen production by HSCF [27], which may be relevant to normal wound healing [2,28-31]. Notwithstanding this, overexpression of the $A_{2A}R$ in fibroblasts has been implicated in the pathogenesis of skin fibrotic malignancies, such as dermal fibrosis and scleroderma, whose patients present excessive collagen deposition in the skin and visceral organs [32].

Controversy however exists about the mechanism(s) underlying the fibrogenic actions of adenosine via $A_{2A}R$ activation, reviewed in [33], mostly because downstream production of cyclic AMP exerts paradoxical effects on collagen production via differential sensitivity of protein kinase A (PKA) and exchange protein activated by cyclic adenosine monophosphate (EPAC) to this intracellular messenger [27,30,34]. Non-canonical, AKT-dependent, Smad2/3-independent signalling, may also explain the paradoxical shift in the effect of increasing concentrations of cyclic AMP on collagen production [26]. Downstream interference with the equilibrium between transcription promoters and repressors of matrix-producing genes have additionally been hypothesized in this context. Activation of $A_{2A}R$ in human dermal fibroblasts suppresses friend leukemia integration-1 (Fli1), which is normally associated with increases in the synthesis of connective tissue growth factor (CTGF/CCN2) and of type I collagen [28].

Interestingly, the pro-fibrotic effect of adenosine resembles the action of common inflammatory mediators (e.g. histamine, bradykinin), which indirectly favour the release of ATP via Panx1 and/or Cx43 containing hemichannels [5,6], but this association has never been explored so far. This prompted us to investigate the putative interplay between $A_{2A}R$ activation and the expression/function of ATP-releasing Panx-1 and Cx43 hemichannels in cultured HSCF, in order to uncover the role of adenosine in human fibroblast cells function *vis a vis* the putative implications of the nucleoside in subcutaneous tissue remodelling, scarring, inflammation, and nociception that may underlie chronic myofascial pain conditions.

Results

Human subcutaneous fibroblasts (HSCF) exhibit increasing amounts of Panx1 and Cx43 proteins along the time of the cells in culture. Cultured human subcutaneous fibroblasts (HSCF) are elongated cells showing a spindle-shape characteristic morphology. Their fibroblastic nature was confirmed by immunocytochemistry. All cells exhibited positive immunoreactivity against type 1 collagen and vimentin, the intermediate protein filament considered a reliable fibroblast-cell marker (Figure 1 Ai-iii).

Previously, we showed that cultured HSCF exhibit strong immunoreactivity against $A_{2A}R$ which co-localizes with the adenosine forming enzyme, ecto-5'-nucleotidase/CD73; yet, these cells lack the ability to inactivate adenosine to inosine as they are essentially devoid of adenosine deaminase (ADA) [27]. By immunofluorescence confocal microscopy and Western blot analysis, we show here that HSCF express Panx1 (~48 kDa) and Cx43 (43 kDa) proteins (*cf.* [5,6]), which content increases with the time and differentiation of the cells in culture (Figure 1B, 1C and 1D). This feature compares with maturation increases in $A_{2A}R$ and ecto-5'-nucleotidase/CD73 immunoreactivity observed previously [27]. It is however, worth noting that Cx43 expression was always higher than that of Panx1 at all culture time points.

Adenosine A_{2A} receptor activation differently affects the expression of Panx1 and Cx43 in human subcutaneous fibroblasts (HSCF). Using two enzymatically-stable adenosine analogues, 5'-(N-ethylcarboxamide)-adenosine (NECA, 300 nM, a non-selective A_2 receptor agonist) and CGS21680 (10 nM, a selective A_{2A} receptor agonist), we show here that activation of A_{2A} receptors in HSCF differently affects the expression of Panx1 and Cx43 (Figure 2). Using immunofluorescence confocal microscopy and Western blot analysis, we detected significant ($P<0.05$) increases in Panx1 immunoreactivity when cultured HSCF were exposure to NECA (300 nM) and CGS21680 (10 nM) for 7 days (Figure 2A and 2Ci), while the opposite was observed regarding the Cx43 protein content (Figure 2B and 2Cii). Involvement of the A_{2A} receptor was confirmed using the selective A_{2A} receptor antagonist, SCH442416 (10 nM), which fully prevented the dual modulation of Panx1 and Cx43 caused by NECA (300 nM) and CGS21680 (10 nM) in HSCF (Figure 2C).

Adenosine A_{2A} receptor activation favours the release of ATP via Panx1-containing hemichannels in cultured human subcutaneous fibroblasts (HSCF). Considering that activation of A_{2A} receptors significantly enhanced the expression of Panx1 hemichannels in HSCF, we set to test the ability of challenged cells to release higher amounts of ATP into the extracellular microenvironment using the luciferin-luciferase bioluminescence assay. Figure 3 shows that 7-day incubations with NECA (300 nM) and CGS21680 (10 nM) significantly ($P<0.05$) increased ATP levels in the culture medium normalized by the number of viable cells (MTT value) in each well. NECA (300 nM; Figure 3A)- and CGS21680 (10 nM; Figure 3B)-induced facilitation of ATP release by HSCF was prevented by application of these compounds together with the selective A_{2A} receptor antagonist, SCH442416 (10 nM; $P<0.05$).

To test whether Panx1-containing hemichannels were involved in the facilitatory effect of CGS2160C (10 nM) on ATP

release by cultured HSCF, the selective A_{2A} receptor agonist was applied together with probenecid (100 μM), a powerful inhibitor of Panx1 hemichannels without any action on connexin-containing channels [35]. As a matter of fact, probenecid (100 μM) mimicked the preventive effect of the A_{2A} receptor antagonist, SCH442416 (10 nM), on CGS21680C (10 nM)-induced facilitation of ATP release by HCSF (Figure 3B).

Blockage of ATP-releasing Panx1 hemichannels prevents the fibrogenic effect of adenosine via A_{2A} receptor activation in human subcutaneous fibroblasts (HSCF). Incubation with CGS 21680 (10 nM, for 7 days) increased (P<0.05) collagen production (Sirius Red assay; Figure 4B) by cultured HSCF without affecting cells growth/viability (MTT assay; Figure 4A), thus discarding any effect of the A_{2A} receptor agonist on cells proliferation, apoptosis and senescence [36]. The fibrogenic effect of CGS 21680 (10 nM) was fully prevented when this compound was applied together with the selective A_{2A} receptor antagonist, SCH442416 (10 nM), as shown previously by our group [27]. We show in Figure 4B that the inhibitory effect of the A_{2A} receptor antagonist was mimicked by blockage of ATP-releasing Panx1 hemichannels with probenecid (100 μM), which on its own decreased collagen production by 10±2% (n=5) only from culture day 21 onwards without affecting HSCFs growth/viability (MTT assay). This feature is in keeping with the increase in Panx1 immunoreactivity along time of the cells in culture (see Figure 1B and 1D).

Using AMP as an adenosine precursor, our group showed previously that the A_{2A} receptor facilitates collagen production by coupling to the adenylyl cyclase (AC) / exchange protein activated by cyclic AMP (EPAC) pathway in HSCF [27]. This hypothesis is strengthen by our results showing that selective inhibition of AC (with SQ22536; 30 μM), as well as inhibition of EPAC (with ESI-09, 10 μM), prevented the facilitatory effect of the selective A_{2A} receptor agonist, CGS 21680 (10 nM), on collagen production by HSCF under similar experimental conditions (Figure 4B).

Adenosine A_{2A} receptor-induced Panx1 overexpression contributes to sustain histamine-induced $[Ca^{2+}]_i$ accumulation in human subcutaneous fibroblasts (HSCF). Mast cell-derived histamine plays an important role in painful fibrotic diseases, and that it may trigger the release of ATP from HSCF via Panx1-containing hemichannels resulting in sustained $[Ca^{2+}]_i$ mobilization [5]. Therefore, we next set to investigate whether A_{2A} receptor-induced Panx1 overexpression affected $[Ca^{2+}]_i$ transients caused by histamine in 7-day cultures where normally cells express little amounts of this protein (see Figure 1B and 1D). Histamine (100 μM) caused a fast (within seconds) $[Ca^{2+}]_i$ rise in cultured HSCF which attained 68±9% (n=11) of the maximal calcium load produced by the calcium ionophore, ionomycin (5 μM; 100% response) (Figure 5A-C). Following this fast intracellular Ca^{2+} mobilization, a slow decay lasting about 2 min was observed; beyond that point, histamine-induced Ca^{2+} influx from the extracellular milieu keeps $[Ca^{2+}]_i$ levels fairly constant until drug washout (Figure 5; *cf.* [5]).

Acute application of the selective A_{2A} receptor agonist, CGS 21680C (10 nM), had a negligible effect on $[Ca^{2+}]_i$ in HSCF when it was applied alone, *i.e.* in the absence of histamine (100 μM; data not shown). The kinetics of $[Ca^{2+}]_i$ transients induced by histamine (100 μM) in HSCF pre-treated with CGS 21680 (10 nM, for 7 days) was kept almost unaltered compared to non-treated cells (Figure 5A-C). Notwithstanding this, manipulation of Panx1 hemichannels permeability, with both probenecid (100 μM; Figure 5A) and the Panx1 mimetic inhibitory peptide, ^{10}Panx (100 μM; Figure 5B)[37], as well as inactivation of extracellular ATP with apyrase (2 U/ml; Figure 5C), significantly (P<0.05) decreased the late component of histamine (100 μM) responses in CGS 21680C (10 nM)-treated cells, while keeping fairly conserved the initial $[Ca^{2+}]_i$ rise. Notably, none of aforementioned drugs affected significantly (P>0.05) histamine (100 μM)-induced $[Ca^{2+}]_i$ transients in non-treated HSCF at this culture stage (Figure 5A-C). Involvement of the A_{2A} receptor was confirmed given that selective blockage of this receptor with SCH442416 (10 nM) largely attenuated (P<0.05) the inhibitory actions of probenecid (100 μM; Figure 5A), ^{10}Panx (100 μM; Figure 5B) and apyrase (2 U/ml; Figure 5C) in the late $[Ca^{2+}]_i$ plateau response to histamine (100 μM) in CGS 21680C (10 nM)-treated cells. Overall, these findings suggest that overexpression of Panx1 hemichannels produced by persistent activation of adenosine A_{2A} receptors may contribute to sustain histamine-induced $[Ca^{2+}]_I$ responses in HSCF by fostering the release of ATP and, thereby, promoting the cooperation between H_1 and P2 receptors [5].

Discussion

Considering that fascia is a mechano-sensitive organ bridging all parts of the body and that it is composed mainly by fibroblasts interacting with large number of neurons operating proprioception and nociception [38,39], one can easily understand that disorganization of this complex cellular network structure may affect health condition in many different ways [40]. Therefore, we foresee that investigating the molecular mechanisms underlying fascial fibroblasts signalling role may contribute to unravel alterations of the whole body system in myofascial pain syndromes, like fibromyalgia.

Data show here for the first time that adenosine, via prolonged activation of high affinity A_{2A} receptors, promotes Panx-1 overexpression while decreasing the amount of Cx43-containing channels in HSCF. The A_{2A} receptor-induced Panx1 overexpression favours the release of large amounts of ATP from these cells, thus creating a vicious cycle that (1) promotes synthesis and extracellular deposition of collagenous extracellular matrix, and (2) contributes to intracellular $[Ca^{2+}]_I$ accumulation in response to common inflammatory mediators (e.g. histamine) (see Figure 6), which may be critical to the pathogenesis of adenosine-induced dermal fibrosis, skin radiation injury and chronic myofascial pain conditions (see below).

Changes in the activity and expression of Cx43 and Panx-1 have been implicated in fibrosis, inflammation and chronic pain [14,18,19,21]. Apparently, these two important components of transmembrane hemichannels exert differential, or even opposite, effects in dermal healing and fibrosis. Hemichannels containing Panx-1 are widely accepted as putative mediators of ATP

translocation to the extracellular milieu in non-excitable cells, including fibroblasts [5,41]. Panx-1 hemichannels have also been increasingly linked to inflammation [42], as they integrate inflammasome activation and promote secretion of pro-inflammatory mediators, like interleukin-1β and histamine [5,20]. The launch of ATP release via Panx-1 hemichannels is additionally involved in neutrophil chemotaxis [43]. These authors showed that autocrine stimulation of ATP-sensitive $P2Y_2$ receptors contributes to the excitatory signals at the front of polarized neutrophils, while causing inhibitory signals at the back of these cells providing that ATP is broken down into adenosine favouring activation of backwardly redistributed A_{2A} receptors [43]. Likewise, the release of ATP via Panx1 hemichannels also plays a role in the development and maintenance of chronic mechanical hypersensitivity [21] and in dermal fibrosis [22].

Notwithstanding the fact that (1) adenosine A_{2A} receptors activation simulates the fibrogenic actions of inflammatory mediators (e.g. histamine, bradykinin) by indirectly favouring ATP release via Panx1 hemichannels [5,6], and that (2) adenosine resulting from the breakdown of released ATP extensively accumulates in the extracellular milieu favouring activation of A_{2A} receptors located in close proximity to the adenosine forming enzyme, ecto-5'-nucleotidase/CD73 [27], this is the first study to show that the nucleoside (via a mechanism involving A_{2A} receptors coupled to the AC/EPAC pathway) participates in a vicious cycle that fosters ATP release, intracellular $[Ca^{2+}]_i$ oscillations and collagen production by HSCF. Thus, upon moderate adenosine A_{2A} receptors activation, Panx-1 upregulation and ATP release from HSCF create an appropriate environment to increase collagen synthesis and to promote inflammation resolution and wound healing [2,27] Coincidently, Panx1 expression and function in HSCF paralleled the amounts of adenosine-forming enzyme, ecto-5'-nucleotidase, and of the A_{2A} receptor in these cells along culture maturation [27]. Though HSCF are relatively free of ADA in their plasma membranes, this adenosine-inactivating enzyme exists in large amounts at the surface of mononuclear cells in association with the multifunctional glycoprotein CD26 which exhibits dipeptidylpeptidase IV (DPP4) activity. Thus, mononuclear inflammatory cell infiltrates carry high surface amounts of CD26–ADA complexes, which may negatively impact on adenosine mediated dermal nociception (via neuronal A_1 receptors) and wound healing (via fibroblast A_{2A}) [44], while increasing inosine-sensitive A_3-mediated actions that are putatively involved in fibromyalgia patients [27,45].

On the other hand, it has been shown that fibroblasts overexpressing adenosine A_{2A} receptors may produce a hostile environment leading to dermal fibrosis [22] and scleroderma [32]. Likewise, upon tissue destruction by ionizing radiation extracellular adenosine accumulation may promote skin fibrosis and scarring via A_{2A} receptors activation [46]. According to our data this might result from allocation of superfluous amounts of Panx1 hemichannels to the plasma membrane leading to unrestrained ATP release, which further increases $[Ca^{2+}]_i$ mobilization, fibroblast cells growth and abnormal collagen deposition through the cooperation between inflammatory mediators (e.g histamine, bradykinin) and P2 purinoceptors activation [5,6]; see also [47,48]. In a recent paper, Panx1 overexpression has been implicated in a P2X7-independent inflammatory mechanism undertaken by activation of the TNF-α/IL-1β pathway in human umbilical vein endothelial cells [49], but involvement of adenosine via A_{2A} receptors activation has not been ruled out. Interestingly, synergism between Panx1-mediated ATP release and ionotropic P2X3/4 purinoceptors activation may mediate skin hypersensitivity commonly occurring in myofascial syndromes [50]. Likewise, an interplay between Panx1-mediated ATP release and P2X7 receptors activation has been shown to modulate human dermal fibroblasts migration and cell surface actin dynamics [51].

Contrariwise, Cx43 levels normally decay one day after skin injury returning to homeostatic levels after wound closure, both in humans and rodents [52]. In view of this, blockage of Cx43 hemichannels has reach phase II clinical trials to accelerate healing of lower limb venous ulcers and diabetic foot lesions by reducing the scar area and the number of infiltrating inflammatory cells, which on their own favour recovery of dermal histoarchitecture and skin mechanical resistance [17]. Though the mechanism by which inhibition of Cx43 channels stimulates wound healing is still controversial, blockage of Cx43 channels increases the proportion of fitted GJs in relation to their ATP-releasing hemichannel counterparts, thus promoting intercellular communication via GJs in HeLa cells [53]. Moreover, acute downregulation of Cx43 at wounded sites tapers inflammatory responses, and increases fibroblasts migration and keratinocytes proliferation [15]. Downmodulation of the expression and function of Cx43 may play a role against chronic neuropathic pain [14], in particular when this protein is overexpressed [18].

In contrast to Panx1 overexpression, we show here that subacute activation of adenosine A_{2A} receptors significantly downregulated Cx43 protein in cultured HSCF by about 50-75%. This finding prevented us to investigate the implication of Cx43 in the facilitatory effect of A_{2A} receptors on ATP release and histamine-induced $[Ca^{2+}]_i$ accumulation by these cells, also because Cx43, unlike Panx1, hemichannels are usually inactive at normal extracellular Ca^{2+} concentrations (e.g. 1.8 mM $CaCl_2$ in the Tyrode's solution) [10]. Though it has been demonstrated that phosphorylation of multiple Cx43 amino-acid residues inhibits hemichannels assembly, gating, turnover and gap junctional communication (see, e.g. [54]), it remains to be elucidated whether this mechanism is implicated in A_{2A} receptor-induced downmodulation of Cx43 in HSCF and if it also affects other regulatory functions of this protein inside these cells. Additionally, implication of these mechanisms in myofascial inflammatory pain modulation is also worth to be investigated in the future. Yet, this venture requires more demanding experimental techniques which are beyond our actual expertise, like fluorescence recovery after photobleaching (FRAP) to follow fluorescent dyes transfer via GJs and/or the use of HSCF co-cultures with sensitive neurons or inflammatory cells, before going to decisive *in vivo* animal models.

In conclusion, this study shows for the first time that extracellular adenosine, via A_{2A} receptors activation, exerts a dual role on Panx1- and Cx43-containing hemichannels expression with significant functional repercussions on ATP release, histamine-induced $[Ca^{2+}]_i$ mobilization and collagen production by HSCF (Figure 6). These findings, contribute to elucidate the mechanisms underlying the fibrogenic effect of the nucleoside in the pathogenesis of dermal malignancies associated with cellular

overexpression of the A_{2A} receptor. Data also strengthen the theory that uncovering the purinergic signalling complexities might unravel novel putative targets to control myofascial syndromes often associated with fascial fibrosis, connective tissue inflammation and pain perception at certain cutaneous trigger points [40].

Methods

Cell cultures

Human fibroblasts were isolated from subcutaneous tissue samples of male tissue donors (35 ± 6 years old, n = 18) with no clinical history of connective tissue disorders. This study and all its procedures were approved by the Ethics Committees of Centro Hospitalar Universitário do Porto (CHUP, University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of University of Porto. All patients signed an informed consent approved by the Ethics Committee of CHUP for use of the biologic material. Regarding deceased tissue donation, the legal frame work allows the "Presumed Consent," stating that residents in Portugal are consenting donors unless the individual previously objected during his or her life. The investigation conformed to the principles outlined in The Code of Ethics of the World Medical Association (Declaration of Helsinki).
Subcutaneous tissues were maintained at 4-6ºC in M-400 transplantation solution (4.190 g/100 mL mannitol, 0.205 g/100 mL KH_2PO_4, 0.970 g/100 mL $K_2HPO_4 \cdot 3H_2O$, 0.112 g/100 mL KCl, and 0.084 g/100 mL $NaHCO_3$, pH 7.4) until used, which was between 2 and 16 hours after being harvested [5,6,27]. Cells were then obtained by the explant technique and cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 2.5 µg/mL of amphotericin B and 100 U/mL of penicillin/streptomycin, at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Medium was replaced twice a week. Primary cultures were maintained until near confluence (~3-4 weeks), then adherent cells were enzymatically released with 0.04% trypsin-EDTA solution plus 0.025% type I collagenase in phosphate-buffered saline (PBS). The resultant cell suspension was plated and maintained in the same conditions mentioned above. All the experiments were performed in the first subculture.

Immunocytochemistry

Human subcutaneous fibroblasts (HSCF) were seeded in chamber slides at a density of $1.0x10^4$ cells/mL and allowed to grow for 7 days in culture. Cultured cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes, washed 3 times in PBS (10 min each), and subsequently incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), 0.1% Triton X, 0.05% NaN_3) for 1 h. Primary antibodies, diluted in blocking buffer II (5% FBS, 0.5% BSA, 0.05% Triton X, 0.05% NaN_3), were applied [rabbit anti-human type I collagen 1:50 (#2150-0020; AbDSerotec, Kidlington, UK); mouse anti-porcine vimentin 1:75 (#M0725; DAKO, Santa Clara, CA, EUA); rabbit anti-human Cx43 1:6000 (#ab11370; Abcam Plc, Cambridge, UK); rabbit anti-human Panx1 1:250 (#710184; Novex, Life Technologies, Carlsbad, CA, EUA)] and the slides incubated overnight at 4ºC. After incubation, cells were washed 3 times in PBS 1X (10 min each). The donkey anti-rabbit Alexa Fluor 488 (#A-21206; 1:1500) and the donkey anti-mouse Alexa Fluor 568 (#A-10037; 1:1500) secondary antibodies (Molecular Probes, Invitrogen, Waltham, MA, USA) were diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton-X) and applied for 1 h protected from light. A last wash was performed with PBS 1X, and glass slides were mounted with VectaShield medium and stored at 4ºC. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity for the secondary antibodies was tested in control experiments in which primary antibodies were omitted. Observations were performed and analysed with an Olympus FV1000 confocal microscope (Tokyo, Japan) [5,6,27].

SDS–PAGE and Western blotting

The methodology used in these experiments was as previously described by our group [5,6,27]. In brief, HSCF were seeded in chamber slides at a density of 6.0 $x10^4$ cells/mL and allowed to grow for 7 and 28 days in culture. Cells were homogenized in a lysis buffer with the following composition: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton-X-100, 0.1% SDS and a protease inhibitor cocktail. Protein content of the samples was evaluated using the Pierce BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Samples were solubilized in SDS reducing buffer (0.125 mM Tris-HCl, 4% SDS, 0.004% bromophenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70ºC for 10 min), subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (MilliPore, Burlington, MA, USA). Protein loads were 75 µg for Panx1 and 30 µg for Cx43. The membranes were blocked for 1 h in Tris buffered saline (TBS: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 + 5% BSA. Membranes were subsequently incubated with rabbit anti-human Panx1 1:200 (#710184; Novex, Life Technologies, Carlsbad, CA, USA) and rabbit anti-human Cx43 1:4000 (#ab11370; Abcam Plc, Cambridge, UK) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 min in 0.1% Tween 20 in TBS and then incubated with donkey anti-rabbit IgG (HRP) 1:70000 (#ab7083; Abcam Plc, Cambridge, UK) for 60 min at room temperature. For normalization purpose, membranes were also incubated with the GAPDH 1:200 (#sc32233; Santa Cruz, Dallas, TX, USA). Membranes were washed three times for 10 min in 0.1% Tween 20 in TBS and then incubated with donkey anti-mouse IgG (HRP) 1:20000 (#ab98799; Abcam Plc, Cambridge, UK) secondary antibody for 60 min at room temperature. Membranes were washed three times for 10 min and antigen-antibody complexes were visualized by chemiluminescence with an ECL reagent using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Gel band image densities were quantified with ImageJ (National Institute of Health, Bethesda, MD, USA).

Cell viability/proliferation and total collagen determination

HSCF were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL and cultured in supplemented DMEM. Viability/proliferation studies included the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, which was performed according to the manufacturer's instructions as previously described [5,6,27]. Collagen determination was performed using the Sirius Red staining assay that stains equally well collagen types I and III [55], which are the two main collagen types existing in the human skin, normally at a ratio of 4:1 [34]. Cell layers were washed twice in PBS before fixation with Bouin's fluid for 1 h. The fixation fluid was removed by suction, and the culture plates were washed by immersion in running tap water for 15 min. Culture dishes were allowed to air dry before adding the Sirius Red dye (Direct Red 80). Cells were stained for 1 h under mild shaking on a microplate shaker. To remove non-bound dye, stained cells were washed with 0.01 N hydrochloric acid and then dissolved in 0.1 N sodium hydroxide for 30 min at room temperature using a microplate shaker. Optical density was measured at 550 nm against 0.1 N sodium hydroxide as blank using a microplate reader spectrometer (Synergy HT, BioTek, Winooski, VT, USA) [55]. Results were expressed as A/well.

Extracellular ATP quantification by bioluminescence

Extracellular ATP was detected with the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science; Penzberg, Germany) using a multidetection microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), as described elsewhere [5,6]. Briefly, cells were cultured for 7 days in the absence or in the presence of 5′-(N-ethylcarboxamide)-adenosine (NECA, 300 nM, a non-selective A_2 receptor agonist) or of CGS21680 (10 nM, a selective $A_{2A}R$ agonist); the two agonists were either applied alone or together with the selective $A_{2A}R$ antagonist, SCH442416 (10 nM). Before measurements, cells were washed twice with Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.2 mM glucose, pH 7.4) at 37 °C and allowed to rest for 30 min.

Measurement of intracellular $[Ca^2]_i$ transients

Changes in intracellular $[Ca^{2+}]_i$ were measured at 37 °C in cells loaded with the calcium-sensitive dye Fluo-4 NW (Fluo-4 NW calcium assay kit; Molecular Probes, Invitrogen, Waltham, MA, USA) using a multidetection microplate reader (Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments, Winooski, VT, USA), as described previously [5,6]. After seeding HSCF cells in flat bottom 96-well plates at a density of 3×10^4 cells/ml, they were allowed to grow for 7 days in the presence and in the absence of the selective $A_{2A}R$ agonist, CGS21680 (10 nM), applied alone or together with the selective $A_{2A}R$ antagonist, SCH442416 (10 nM). Before $[Ca^{2+}]_i$ measurements, cells were washed twice with Tyrode's solution to remove any traces of culture media and unloaded Ca^{2+} indicator. Fluorescence was excited at 485/20 nm, and emission was measured at 528/20 nm. Calcium measurements were calibrated to the maximal calcium load produced by ionomycin (5 μM; 100% response)[56].

Materials and reagents

Amphotericin B, bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), ethylene diaminetetraacetic acid (EDTA), fetal bovine serum (FBS), trypsin-EDTA solution plus, Direct Red 80, ethylene-bis(oxyethylenenitrilo)tetra acetic acid (EGTA)**,** 2-(4-imidazolyl)ethylamine (Histamine), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT), 5′-(N ethylcarboxamide) adenosine (NECA), penicillin/streptomycin, phosphate buffered saline system (PBS), and type I collagenase, 2-(2-Furanyl)-7-[3-(4-methoxyphenyl) propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin- 5-amine (SCH 442416), and 4-[2-[[6-Amino-9-(Nethyl-β-D-ribofuranuronamidosyl)- 9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS 21680), α-[2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-b-oxo-3-isoxazolepropanenitrile (ESI-09) and 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine, (SQ22,536) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dimethylsulphoxide (DMSO) and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Probenecid, ^{10}Panx, 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo [4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH442416), were obtained from Tocris Cookson Inc (Southampton, UK.). Bouin liquor was supplied by PanReac AppliChem ITW Reagents (Barcelona, Spain). Ionomycin was acquired from Abcam (Cambridge, UK). CGS 21680, NECA, SCH 442416, probenecid, ESI-09 and SQ22,536 were diluted in dimethyl sulfoxide (DMSO); all other drugs were prepared in distilled water. Regarding solutions storage (as frozen aliquots at -20ºC) and dilution, pH control and DMSO (maximum 0.05% v/v) testing, we followed that described in previous publications from our group [5,6,27].

Presentation of data and statistical analysis

Data are expressed as mean ± SD from an n number of experiments/cells/individuals. Statistical analysis was carried out using Graph Pad Prism 9.1.0 software (La Jolla, CA, USA). One-way ANOVA, either corrected or uncorrected for multiple comparisons using the Bonferroni's or the Fisher's LSD tests, respectively; few outliers were identified using the ROUT method with a Q = 1%. $P<0.05$ (two-tailed) values were considered statistically significant.

References

1 Correa-Gallegos, D. *et al.* Patch repair of deep wounds by mobilized fascia. *Nature* **576**, 287-292, doi:10.1038/s41586-019-1794-y (2019).

2 Cronstein, B. Adenosine receptors and fibrosis: A translational review. *F1000 biology reports* **3**, 21, doi:10.3410/B3-21 (2011).

3 Sawynok, J. Adenosine receptor targets for pain. *Neuroscience* **338**, 1-18, doi:https://doi.org/10.1016/j.neuroscience.2015.10.031 (2016).

4 Langevin, H. M., Cornbrooks, C. J. & Taatjes, D. J. Fibroblasts form a body-wide cellular network. *Histochem Cell Biol* **122**, 7-15, doi:10.1007/s00418-004-0667-z (2004).

5 Pinheiro, A. R. *et al.* Histamine induces ATP release from human subcutaneous fibroblasts, via pannexin-1 hemichannels, leading to Ca2+ mobilization and cell proliferation. *J Biol Chem* **288**, 27571-27583, doi:10.1074/jbc.M113.460865 (2013).

6 Pinheiro, A. R. *et al.* Bradykinin-induced Ca2+ signaling in human subcutaneous fibroblasts involves ATP release via hemichannels leading to P2Y12 receptors activation. *Cell Commun Signal* **11**, 70-70, doi:10.1186/1478-811X-11-70 (2013).

7 Plikus, M. V. *et al.* Fibroblasts: Origins, definitions, and functions in health and disease. *Cell* **184**, 3852-3872, doi:10.1016/j.cell.2021.06.024 (2021).

8 Lu, D., Soleymani, S., Madakshire, R. & Insel, P. A. ATP released from cardiac fibroblasts via connexin hemichannels activates profibrotic P2Y2 receptors. *The FASEB Journal* **26**, 2580-2591, doi:https://doi.org/10.1096/fj.12-204677 (2012).

9 Willebrords, J., Maes, M., Crespo Yanguas, S. & Vinken, M. Inhibitors of connexin and pannexin channels as potential therapeutics. *Pharmacology & Therapeutics* **180**, 144-160, doi:https://doi.org/10.1016/j.pharmthera.2017.07.001 (2017).

10 Fasciani, I. *et al.* Regulation of connexin hemichannel activity by membrane potential and the extracellular calcium in health and disease. *Neuropharmacology* **75**, 479-490, doi:https://doi.org/10.1016/j.neuropharm.2013.03.040 (2013).

11 Zhang, K. *et al.* The mutual interplay of redox signaling and connexins. *Journal of Molecular Medicine*, doi:10.1007/s00109-021-02084-0 (2021).

12 Montgomery, J. *et al.* The connexin 43 carboxyl terminal mimetic peptide αCT1 prompts differentiation of a collagen scar matrix in humans resembling unwounded skin. *Faseb j* **35**, e21762, doi:10.1096/fj.202001881R (2021).

13 Wang, C. M., Lincoln, J., Cook, J. E. & Becker, D. L. Abnormal Connexin Expression Underlies Delayed Wound Healing in Diabetic Skin. *Diabetes* **56**, 2809, doi:10.2337/db07-0613 (2007).

14 Morioka, N., Nakamura, Y., Zhang, F. F., Hisaoka-Nakashima, K. & Nakata, Y. Role of Connexins in Chronic Pain and Their Potential as Therapeutic Targets for Next-Generation Analgesics. *Biological and Pharmaceutical Bulletin* **42**, 857-866, doi:10.1248/bpb.b19-00195 (2019).

15 Mori, R., Power, K. T., Wang, C. M., Martin, P. & Becker, D. L. Acute downregulation of connexin43 at wound sites leads to a reduced inflammatory response, enhanced keratinocyte proliferation and wound fibroblast migration. *Journal of Cell Science* **119**, 5193, doi:10.1242/jcs.03320 (2006).

16 Chekeni, F. B. *et al.* Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature* **467**, 863-867, doi:10.1038/nature09413 (2010).

17 Laird, D. W. & Lampe, P. D. Therapeutic strategies targeting connexins. *Nat Rev Drug Discov* **17**, 905-921, doi:10.1038/nrd.2018.138 (2018).

18 Chen, M. J. *et al.* Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. *Glia* **60**, 1660-1670, doi:10.1002/glia.22384 (2012).

19 Li, W. *et al.* Connexin 43 Hemichannel as a Novel Mediator of Sterile and Infectious Inflammatory Diseases. *Sci Rep* **8**, 166, doi:10.1038/s41598-017-18452-1 (2018).

20 Yeung, A. K., Patil, C. S. & Jackson, M. F. Pannexin-1 in the CNS: Emerging concepts in health and disease. *J Neurochem* **154**, 468-485, doi:10.1111/jnc.15004 (2020).

21 Weaver, J. L. *et al.* Hematopoietic pannexin 1 function is critical for neuropathic pain. *Scientific reports* **7**, 42550, doi:10.1038/srep42550 (2017).

22 Feig, J. L. *et al.* The antiviral drug tenofovir, an inhibitor of Pannexin-1-mediated ATP release, prevents liver and skin fibrosis by downregulating adenosine levels in the liver and skin. *PLOS ONE* **12**, e0188135, doi:10.1371/journal.pone.0188135 (2017).
23 Goldman, N., Chandler-Militello, D., Langevin, H. M., Nedergaard, M. & Takano, T. Purine receptor mediated actin cytoskeleton remodeling of human fibroblasts. *Cell Calcium* **53**, 297-301, doi:https://doi.org/10.1016/j.ceca.2013.01.004 (2013).
24 Langevin, H. M. *et al.* Fibroblast cytoskeletal remodeling induced by tissue stretch involves ATP signaling. *J Cell Physiol* **228**, 1922-1926, doi:10.1002/jcp.24356 (2013).
25 Burnstock, G. Purinergic Signalling: Therapeutic Developments. *Frontiers in Pharmacology* **8**, doi:10.3389/fphar.2017.00661 (2017).
26 Perez-Aso, M., Fernandez, P., Mediero, A., Chan, E. S. & Cronstein, B. N. Adenosine 2A receptor promotes collagen production by human fibroblasts via pathways involving cyclic AMP and AKT but independent of Smad2/3. *Faseb j* **28**, 802-812, doi:10.1096/fj.13-241646 (2014).
27 Herman-de-Sousa, C. *et al.* Opposing Effects of Adenosine and Inosine in Human Subcutaneous Fibroblasts May Be Regulated by Third Party ADA Cell Providers. *Cells* **9**, doi:10.3390/cells9030651 (2020).
28 Chan, E. S. *et al.* Adenosine A(2A) receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. *Arthritis Res Ther* **15**, R58, doi:10.1186/ar4229 (2013).
29 Perez-Aso, M., Chiriboga, L. & Cronstein, B. N. Pharmacological blockade of adenosine A2A receptors diminishes scarring. *The FASEB Journal* **26**, 4254-4263, doi:https://doi.org/10.1096/fj.12-209627 (2012).
30 Yokoyama, U. *et al.* The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci U S A* **105**, 6386-6391, doi:10.1073/pnas.0801490105 (2008).
31 Perez-Aso, M. & Mediero, A. Adenosine A2A receptor (A(2A)R) is a fine-tune regulator of the collagen1:collagen3 balance. *Purinergic Signal* **9**, doi:10.1007/s11302-013-9368-1 (2013).
32 Lazzerini, P. E. *et al.* Adenosine A2A receptor activation stimulates collagen production in sclerodermic dermal fibroblasts either directly and through a cross-talk with the cannabinoid system. *J Mol Med (Berl)* **90**, 331-342, doi:10.1007/s00109-011-0824-5 (2012).
33 Shaikh, G. & Cronstein, B. Signaling pathways involving adenosine A2A and A2B receptors in wound healing and fibrosis. *Purinergic Signal* **12**, 191-197, doi:10.1007/s11302-016-9498-3 (2016).
34 Perez-Aso, M., Mediero, A. & Cronstein, B. N. Adenosine A2A receptor (A2AR) is a fine-tune regulator of the collagen1:collagen3 balance. *Purinergic Signal* **9**, 573-583, doi:10.1007/s11302-013-9368-1 (2013).
35 Silverman, W., Locovei, S. & Dahl, G. Probenecid, a gout remedy, inhibits pannexin 1 channels. *Am J Physiol Cell Physiol* **295**, C761-767, doi:10.1152/ajpcell.00227.2008 (2008).
36 Ahsan, M. K. & Mehal, W. Z. Activation of adenosine receptor A2A increases HSC proliferation and inhibits death and senescence by down-regulation of p53 and Rb. *Frontiers in Pharmacology* **5**, doi:10.3389/fphar.2014.00069 (2014).
37 Wang, J., Ma, M., Locovei, S., Keane, R. W. & Dahl, G. Modulation of membrane channel currents by gap junction protein mimetic peptides: size matters. *Am J Physiol Cell Physiol* **293**, C1112-1119, doi:10.1152/ajpcell.00097.2007 (2007).
38 Bordoni, B. & Zanier, E. Understanding Fibroblasts in Order to Comprehend the Osteopathic Treatment of the Fascia. *Evid Based Complement Alternat Med* **2015**, 860934, doi:10.1155/2015/860934 (2015).
39 Kumka, M. & Bonar, J. Fascia: a morphological description and classification system based on a literature review. *J Can Chiropr Assoc* **56**, 179-191 (2012).
40 Adstrum, S., Hedley, G., Schleip, R., Stecco, C. & Yucesoy, C. A. Defining the fascial system. *Journal of Bodywork and Movement Therapies* **21**, 173-177, doi:https://doi.org/10.1016/j.jbmt.2016.11.003 (2017).
41 Penuela, S., Gehi, R. & Laird, D. W. The biochemistry and function of pannexin channels. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1828**, 15-22, doi:https://doi.org/10.1016/j.bbamem.2012.01.017 (2013).
42 Kameritsch, P. & Pogoda, K. The Role of Connexin 43 and Pannexin 1 During Acute Inflammation. *Front Physiol* **11**, 594097-594097, doi:10.3389/fphys.2020.594097 (2020).
43 Bao, Y., Chen, Y., Ledderose, C., Li, L. & Junger, W. G. Pannexin 1 Channels Link Chemoattractant Receptor Signaling to Local Excitation and Global Inhibition Responses at the Front and Back of Polarized Neutrophils*. *Journal of Biological Chemistry* **288**, 22650-22657, doi:https://doi.org/10.1074/jbc.M113.476283 (2013).

44 Guieu, R. *et al.* High cell surface CD26-associated activities and low plasma adenosine concentration in fibromyalgia. *Ann Rheum Dis* **71**, 1427-1428, doi:10.1136/annrheumdis-2011-201174 (2012).
45 Fais, A. *et al.* Purine metabolites in fibromyalgia syndrome. *Clinical Biochemistry* **46**, 37-39, doi:https://doi.org/10.1016/j.clinbiochem.2012.09.009 (2013).
46 Perez-Aso, M., Mediero, A., Low, Y. C., Levine, J. & Cronstein, B. N. Adenosine A2A receptor plays an important role in radiation-induced dermal injury. *Faseb j* **30**, 457-465, doi:10.1096/fj.15-280388 (2016).
47 Cronstein, B. N. & Sitkovsky, M. Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. *Nat Rev Rheumatol* **13**, 41-51, doi:10.1038/nrrheum.2016.178 (2017).
48 Chan, E. S. *et al.* Adenosine A2A receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum* **54**, 2632-2642, doi:10.1002/art.21974 (2006).
49 Yang, Y. *et al.* Endothelial Pannexin 1 Channels Control Inflammation by Regulating Intracellular Calcium. *J Immunol* **204**, 2995-3007, doi:10.4049/jimmunol.1901089 (2020).
50 Harcha, P. A. *et al.* Pannexin-1 Channels Are Essential for Mast Cell Degranulation Triggered During Type I Hypersensitivity Reactions. *Frontiers in Immunology* **10**, doi:10.3389/fimmu.2019.02703 (2019).
51 Flores-Muñoz, C. *et al.* Restraint of Human Skin Fibroblast Motility, Migration, and Cell Surface Actin Dynamics, by Pannexin 1 and P2X7 Receptor Signaling. *International Journal of Molecular Sciences* **22**, 1069 (2021).
52 Lampe, P. D. *et al.* Cellular interaction of integrin alpha3beta1 with laminin 5 promotes gap junctional communication. *J Cell Biol* **143**, 1735-1747, doi:10.1083/jcb.143.6.1735 (1998).
53 Rhett, J., Jourdan, J. & Gourdie, R. Rhett JM, Jourdan J, Gourdie RG.Connexin 43 connexon to gap junction transition is regulated by zonula occludens-1. Mol Biol Cell 22:1516-1528. *Molecular biology of the cell* **22**, 1516-1528, doi:10.1091/mbc.E10-06-0548 (2011).
54 Li, H., Spagnol, G., Zheng, L., Stauch, K. L. & Sorgen, P. L. Regulation of Connexin43 Function and Expression by Tyrosine Kinase 2. *J Biol Chem* **291**, 15867-15880, doi:10.1074/jbc.M116.727008 (2016).
55 Tullberg-Reinert, H. & Jundt, G. In situ measurement of collagen synthesis by human bone cells with a Sirius Red-based colorimetric microassay: Effects of transforming growth factor β2 and ascorbic acid 2-phosphate. *Histochemistry and Cell Biology* **112**, 271-276, doi:10.1007/s004180050447 (1999).
56 Henriksen, Z., Hiken, J. F., Steinberg, T. H. & Jørgensen, N. R. The predominant mechanism of intercellular calcium wave propagation changes during long-term culture of human osteoblast-like cells. *Cell Calcium* **39**, 435-444, doi:https://doi.org/10.1016/j.ceca.2006.01.012 (2006).

Acknowledgments

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects UID/BIM/4308/2016, UID/BIM/4308/2019 and UIDB/04308/2020). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. CHS was in receipt of a PhD Studentship from NORTE-08-5369-FSE-000011, application NORTE-69-2015-15 co-funded by FSE – Fundo Social Europeu through NORTE2020 – Programa Operacional Regional do Norte. The authors wish to thank Mrs. Helena Costa e Silva, Helena Oliveira and Belmira Silva for their valuable technical assistance.

Author contributions statement

Conceptualization, C.H.-d.-S., P.C.-d.-S.; methodology, C.H.-d.-S., R.P.S., M.A.C., F.F., S.R., P.C.-d.-S.; formal analysis, C.H.-d.-S., R.P.S., M.A.C., F.F., P.C.-d.-S; investigation, C.H.-d.-S., R.P.S., M.A.C., F.F., P.C.-d.-S.; resources, P.C.-d.-S.; data curation, C.H.-d.-S., M.A.C., R.P.S., S.R., F.F.; writing—original draft preparation, C.H.-d.-S., P.C.-d.-S.; writing—review & editing, C.H.-d.-S., M.A.C., R.P.S., F.F., S.R. and P.C.-d.-S.; supervision, M.A.C., P.C.-d.-S.; project administration, P.C.-d.-S.; funding acquisition, P.C.-d.-S. All authors have read and agreed to this version of the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1. Human subcutaneous fibroblasts (HSCF) exhibit increasing amounts of Panx1 and Cx43 proteins along the time of the cells in culture. Shown are confocal micrographs of HSCF stained against (A) type I collagen (i) and vimentin (ii), (B) Panx1 and (C) Cx43 obtained at culture days 7 (i) and 28 (ii). Nuclei are stained in blue with DAPI. Micrographs are representative of at least three different individuals and were obtained with a laser scanning confocal microscope using the same acquisition settings. Bar scale: 50 µm. In D, shown are representative immunoblots to document the relative amounts of Panx1 (Di) and Cx43 (Dii) normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in HSCF cultured for 7 and 28 days. Each bar represents pooled data from three different individuals; three replicas were performed in each individual experiment. Vertical bars represent SD. **P*<0.05 (one-way ANOVA) represent significant differences compared to culture day 7.

Figure 2. Adenosine A_{2A} receptor activation differently affects the expression of Panx1 and Cx43 in human subcutaneous fibroblasts (HSCF). Panels A and B, show the immunoreactivity against Panx1 and Cx43, respectively, exhibited by HSCF cultured for 7 days either in the absence (i) or in the presence of NECA (300 nM, ii) or CGS21680C (10 nM, iv); reversion of the NECA (300 nM) effect in the presence of the A_{2A} receptor antagonist, SCH442416 (10 nM, iii), is also shown for comparison. Nuclei are stained in blue with DAPI. Micrographs are representative of at least three different individuals and were obtained with a laser scanning confocal microscope using the same acquisition settings. Bar scale: 50 µm. Panel C, show representative immunoblots to document the relative expression of Panx1 (Ci) and Cx43 (Cii) normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in 7-day HSCF cultures. Each bar represents pooled data from three to five distinct individuals; three replicas were made in each individual experiment. Vertical bars represent SD. **P*<0.05 and ***P*<0.01 (one-way ANOVA) represent significant differences compared to control (CTRL) conditions; ns, non-significant.

Figure 3. Adenosine A_{2A} receptor activation favours the release of ATP via Panx1-containing hemichannels in cultured human subcutaneous fibroblasts (HSCF). Panels A and B, show the amount of ATP in the culture medium normalized by the number of viable cells (MTT value) per well when HSCF were cultured for 7 days in control conditions (CTRL) or in the presence of NECA (300 nM, A) and CGS21680C (10 nM, B); the effects of the A_{2A} receptor antagonist, SCH442416 (10 nM), and of the Panx1 hemichannels blocker, probenecid (PBN, 100µM), are also shown for comparison. Each bar represents pooled data from three to five distinct individuals; three replicas were made in each individual experiment. Vertical bars represent SD. ***P*<0.01 and ****P*<0.001 (one-way ANOVA) represent significant differences compared to control (CTRL) conditions; ns, non-significant.

Figure 4. Blockage of ATP-releasing Panx1 hemichannels prevents the pro-fibrotic effect of adenosine via A_{2A} receptor activation on 7-day cultures of human subcutaneous fibroblasts (HSCF). The adenosine A_{2A} receptor agonist, CGS21680 (10 nM), increases collagen production (Sirius Red staining, panel B) while having no effect on HSCF growth (MTT assay, panel A). The pro-fibrotic effect of CGS21680 (10 nM) was prevented upon blockage of Panx1 hemichannels with probenecid (PBN, 100µM), as well as by inhibiting adenylate cyclase and the exchange protein activated by cyclic AMP (EPAC) with SQ22536 (30 µM) and ESI-09 (10 µM), respectively. Boxes and whiskers represent pooled data from five different individuals; 4–6 replicas were performed for each individual. Black dots represent outliers located outside the 90% confidence interval. *****P*<0.0001 (one-way ANOVA) represent significant differences compared either to control (CTRL) conditions or to the effect of CGS21680 in the same set of experiments.

Figure 5. Adenosine A_{2A} receptor-induced Panx1 overexpression contributes to sustain intracellular $[Ca^{2+}]_i$ accumulation caused by histamine (Hist) in human subcutaneous fibroblasts (HSCF). Shown are fluorescence $[Ca^{2+}]_i$ transients subsequent to Hist (100 µM) application in HSCF grown in culture for 7 days either in control conditions or in the presence of the selective A_{2A} receptor agonist, CGS21680C (10 nM); treatment with CGS21680C (10 nM) plus the A_{2A} receptor antagonist, SCH442426 (10 nM), is also shown for comparison. The effect of Hist (100 µM) was tested in the presence of inhibitors of Panx1-containing hemichannels, namely probenecid (PBN, 100 µM, A) and ^{10}Panx (100 µM, B), as well as of the ATP hydrolysing enzyme, apyrase (2 U/mL, C). Cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW (see "Experimental Procedures"); $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µm; 100% response). None of the inhibitors significantly changed baseline fluorescence when applied alone. Each point represents pooled data from n experiments (shown in brackets). Vertical bars represent SD and are shown when they exceed the symbols in size. **P*<0.05 (one-way ANOVA) represent significant differences compared to the effect of Hist alone obtained in the same culture conditions.

Figure 6. Adenosine A_{2A} receptors activation exerts a dual role in the expression of Panx1- and Cx43-containing hemichannels with significant functional repercussions on ATP release, histamine-induced $[Ca^{2+}]_i$ mobilization and collagen production by HSCF, which might be critical to the pathogenesis of highly-disabling myofascial inflammatory syndromes.

Figures

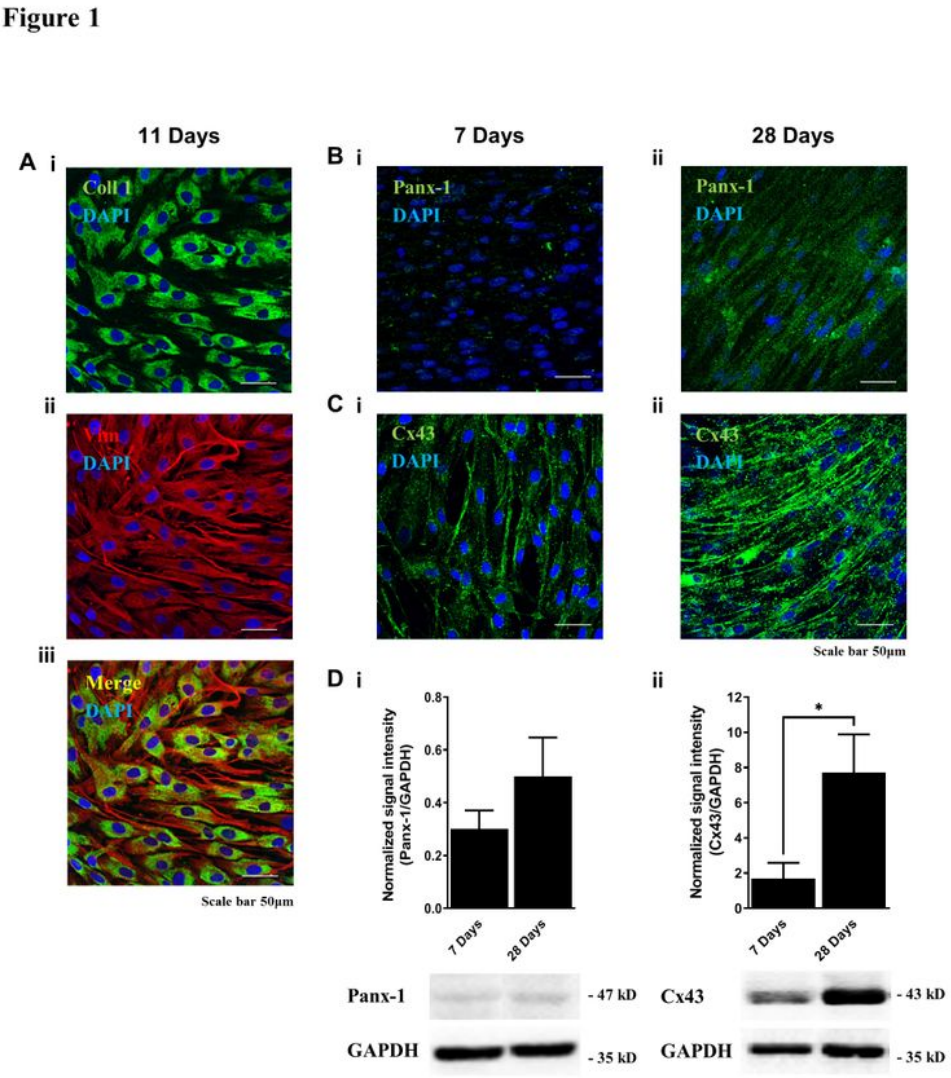

Figure 1

Human subcutaneous fibroblasts (HSCF) exhibit increasing amounts of Panx1 and Cx43 proteins along the time of the cells in culture. Shown are confocal micrographs of HSCF stained against (A) type I collagen (i) and vimentin (ii), (B) Panx1 and (C) Cx43 obtained at culture days 7 (i) and 28 (ii). Nuclei are

stained in blue with DAPI. Micrographs are representative of at least three different individuals and were obtained with a laser scanning confocal microscope using the same acquisition settings. Bar scale: 50 µm. In D, shown are representative immunoblots to document the relative amounts of Panx1 (Di) and Cx43 (Dii) normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in HSCF cultured for 7 and 28 days. Each bar represents pooled data from three different individuals; three replicas were performed in each individual experiment. Vertical bars represent SD. *P<0.05 (one-way ANOVA) represent significant differences compared to culture day 7.

Figure 2

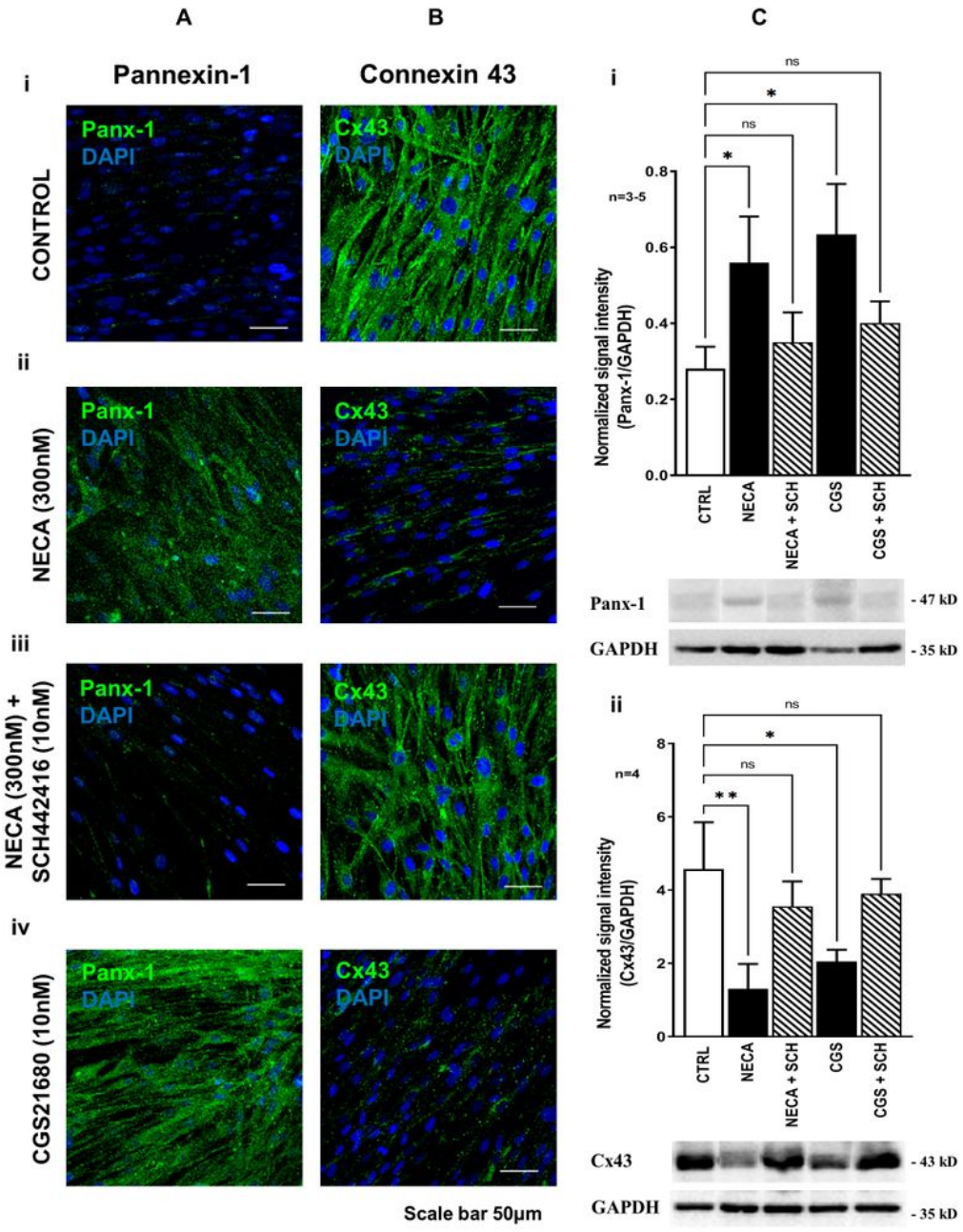

Figure 2

Adenosine A2A receptor activation differently affects the expression of Panx1 and Cx43 in human subcutaneous fibroblasts (HSCF). Panels A and B, show the immunoreactivity against Panx1 and Cx43, respectively, exhibited by HSCF cultured for 7 days either in the absence (i) or in the presence of NECA (300 nM, ii) or CGS21680C (10 nM, iv); reversion of the NECA (300 nM) effect in the presence of the A2A receptor antagonist, SCH442416 (10 nM, iii), is also shown for comparison. Nuclei are stained in blue with DAPI. Micrographs are representative of at least three different individuals and were obtained with a laser scanning confocal microscope using the same acquisition settings. Bar scale: 50 μm. Panel C, show representative immunoblots to document the relative expression of Panx1 (Ci) and Cx43 (Cii) normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in 7-day HSCF cultures. Each bar represents pooled data from three to five distinct individuals; three replicas were made in each individual experiment. Vertical bars represent SD. *P<0.05 and **P<0.01 (one-way ANOVA) represent significant differences compared to control (CTRL) conditions; ns, non-significant.

Figure 3

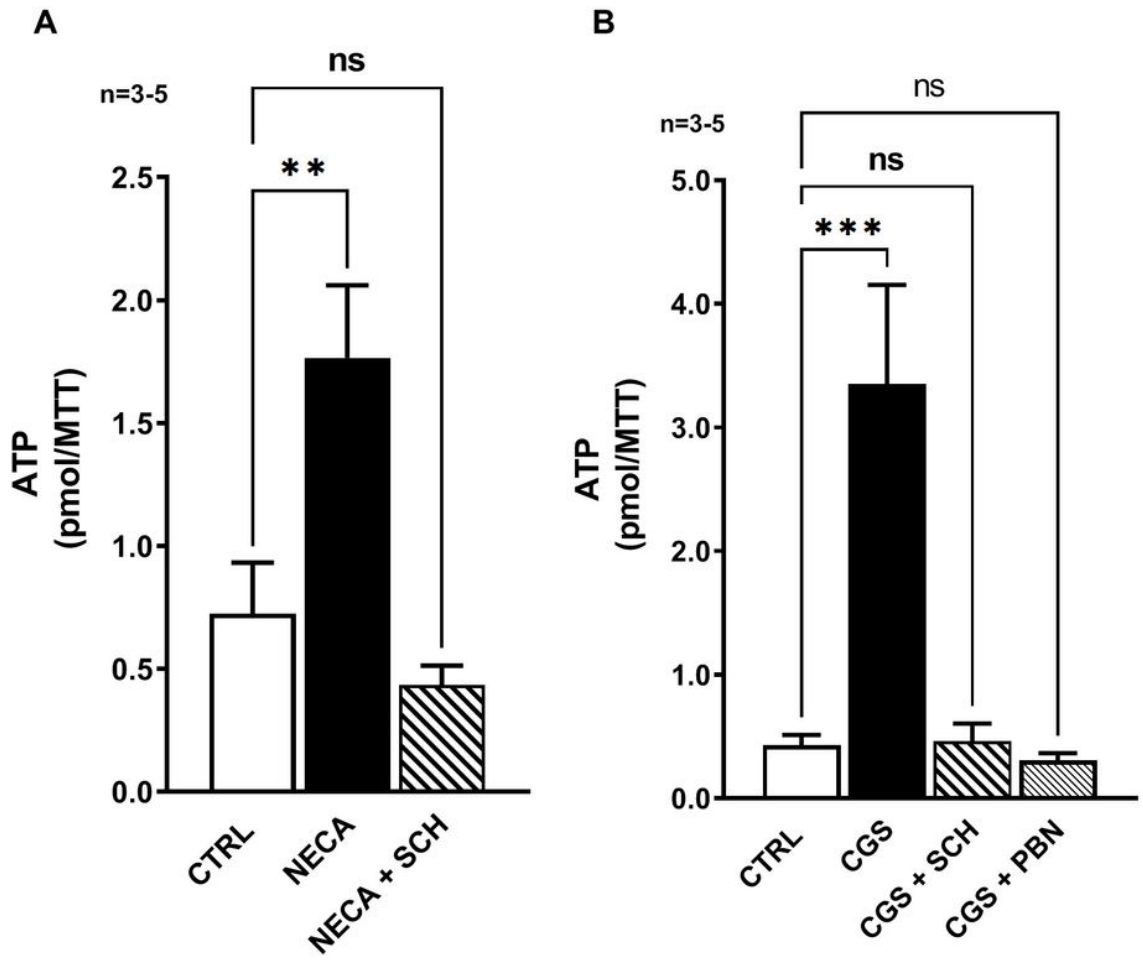

Figure 3

Adenosine A2A receptor activation favours the release of ATP via Panx1-containing hemichannels in cultured human subcutaneous fibroblasts (HSCF). Panels A and B, show the amount of ATP in the culture medium normalized by the number of viable cells (MTT value) per well when HSCF were cultured for 7 days in control conditions (CTRL) or in the presence of NECA (300 nM, A) and CGS21680C (10 nM, B); the effects of the A2A receptor antagonist, SCH442416 (10 nM), and of the Panx1 hemichannels blocker, probenecid (PBN, 100μM), are also shown for comparison. Each bar represents pooled data from three to five distinct individuals; three replicas were made in each individual experiment. Vertical bars represent SD. **P<0.01 and ***P<0.001 (one-way ANOVA) represent significant differences compared to control (CTRL) conditions; ns, non-significant.

Figure 4

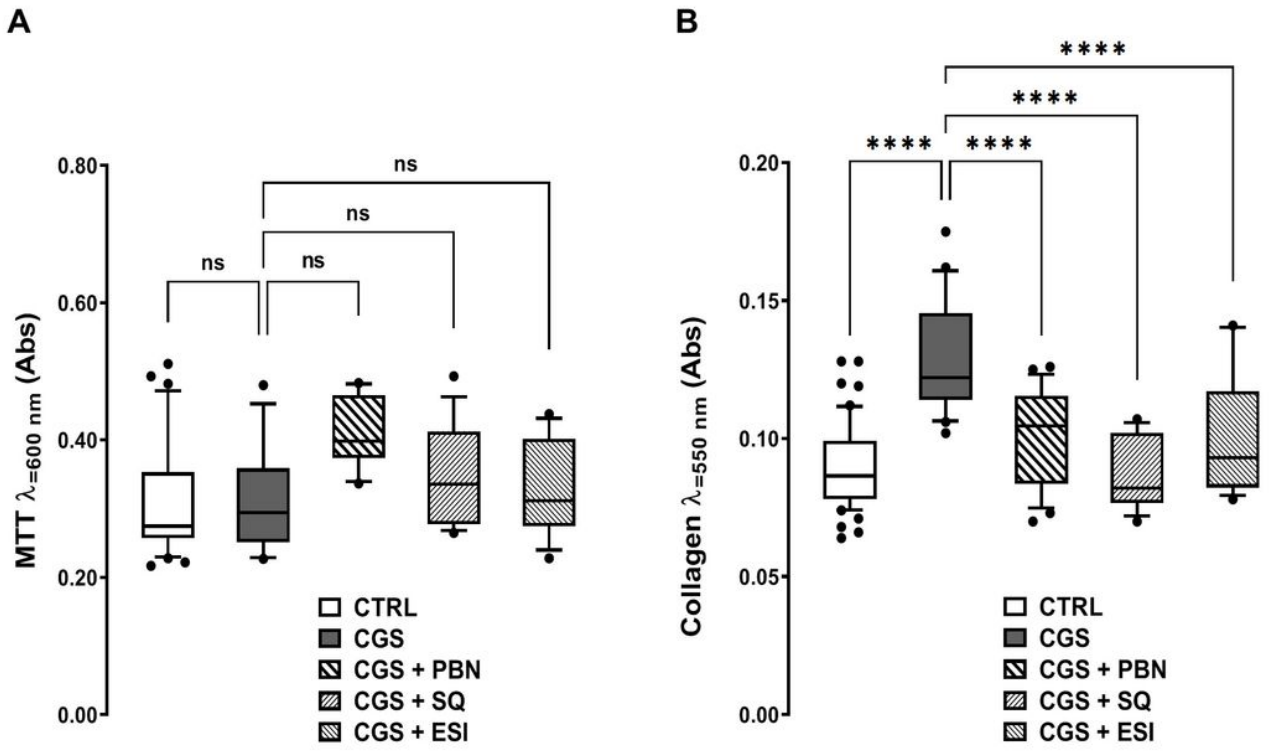

Figure 4

Blockage of ATP-releasing Panx1 hemichannels prevents the pro-fibrotic effect of adenosine via A2A receptor activation on 7-day cultures of human subcutaneous fibroblasts (HSCF). The adenosine A2A receptor agonist, CGS21680 (10 nM), increases collagen production (Sirius Red staining, panel B) while having no effect on HSCF growth (MTT assay, panel A). The pro-fibrotic effect of CGS21680 (10 nM) was prevented upon blockage of Panx1 hemichannels with probenecid (PBN, 100μM), as well as by inhibiting adenylate cyclase and the exchange protein activated by cyclic AMP (EPAC) with SQ22536 (30 μM) and ESI-09 (10 μM), respectively. Boxes and whiskers represent pooled data from five different individuals; 4–6 replicas were performed for each individual. Black dots represent outliers located outside the 90% confidence interval. ****P<0.0001 (one-way ANOVA) represent significant differences compared either to control (CTRL) conditions or to the effect of CGS21680 in the same set of experiments.

Figure 5

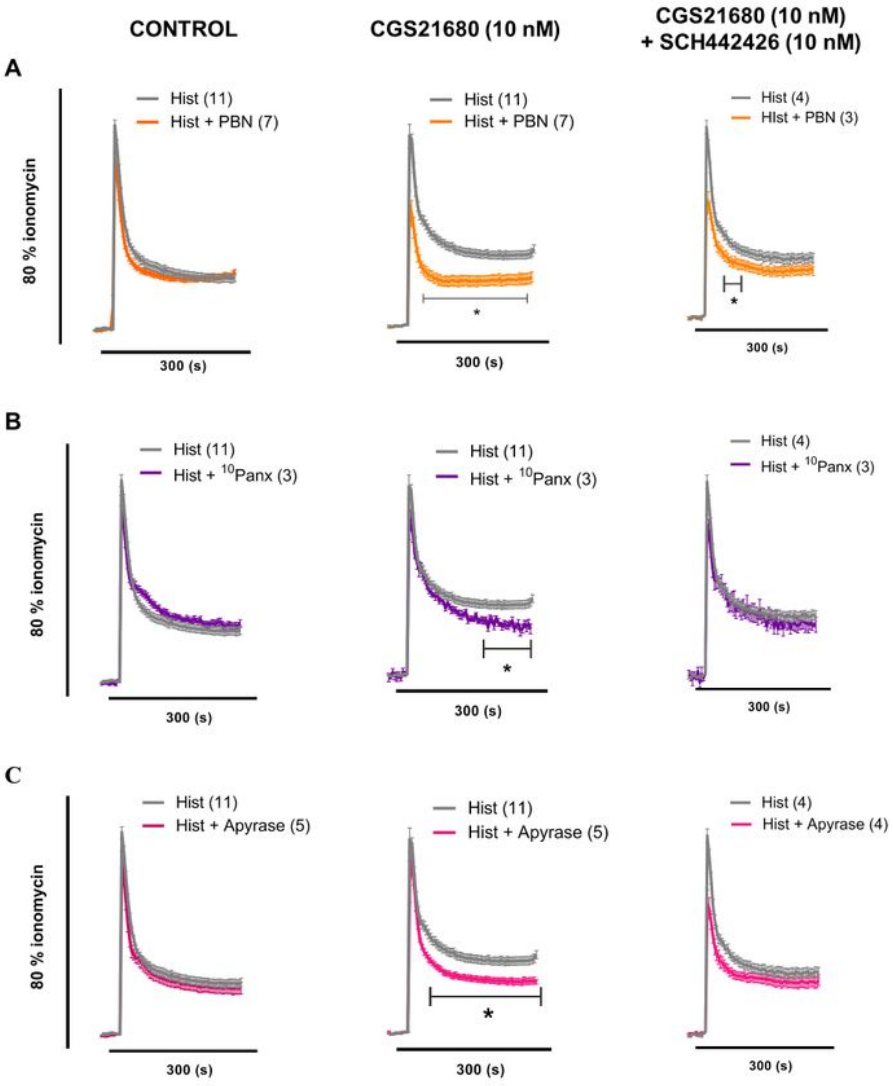

Figure 5

Adenosine A2A receptor-induced Panx1 overexpression contributes to sustain intracellular [Ca2+]i accumulation caused by histamine (Hist) in human subcutaneous fibroblasts (HSCF). Shown are fluorescence [Ca2+]i transients subsequent to Hist (100 μM) application in HSCF grown in culture for 7 days either in control conditions or in the presence of the selective A2A receptor agonist, CGS21680C (10 nM); treatment with CGS21680C (10 nM) plus the A2A receptor antagonist, SCH442426 (10 nM), is also

shown for comparison. The effect of Hist (100 μM) was tested in the presence of inhibitors of Panx1-containing hemichannels, namely probenecid (PBN, 100 μM, A) and 10Panx (100 μM, B), as well as of the ATP hydrolysing enzyme, apyrase (2 U/mL, C). Cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW (see “Experimental Procedures”); [Ca2+]i transients were calibrated to the maximal calcium load produced by ionomycin (5 μm; 100% response). None of the inhibitors significantly changed baseline fluorescence when applied alone. Each point represents pooled data from n experiments (shown in brackets). Vertical bars represent SD and are shown when they exceed the symbols in size. *P<0.05 (one-way ANOVA) represent significant differences compared to the effect of Hist alone obtained in the same culture conditions.

Figure 6

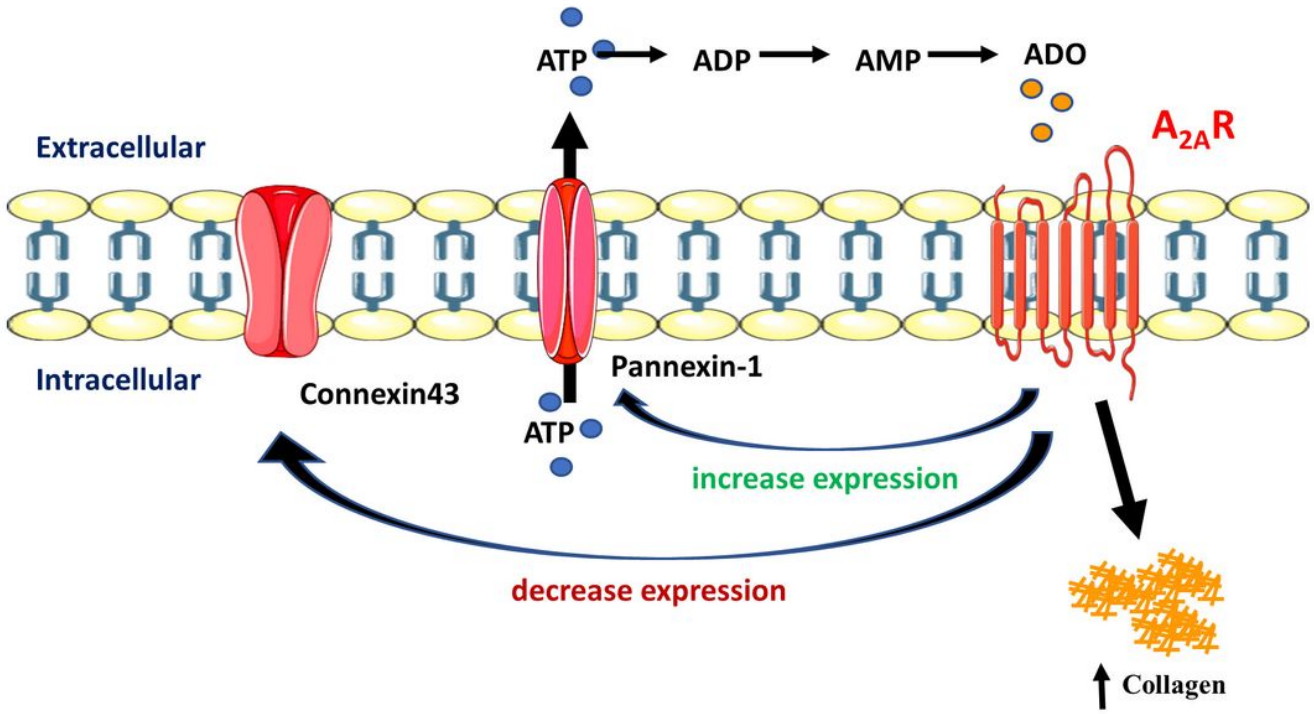

Figure 6

Adenosine A2A receptors activation exerts a dual role in the expression of Panx1- and Cx43-containing hemichannels with significant functional repercussions on ATP release, histamine-induced [Ca2+]i mobilization and collagen production by HSCF, which might be critical to the pathogenesis of highly-disabling myofascial inflammatory syndromes.