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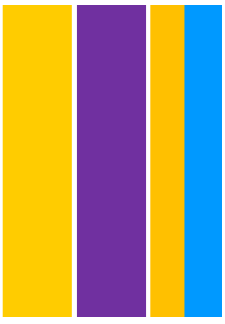
FARMACOLOGIA E TOXICOLOGIA – EXPERIMENTAIS E CLÍNICAS

Mechanisms behind the pro-fibrotic role of adenosine in human subcutaneous fibroblasts and its contribution to myofascial pathological conditions

Carina Talice Stübe Herman de Sousa

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2021



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MECHANISMS BEHIND THE PRO-FIBROTIC ROLE OF ADENOSINE IN HUMAN SUBCUTANEOUS FIBROBLASTS AND ITS CONTRIBUTION TO MYOFASCIAL PATHOLOGICAL CONDITIONS

Dissertação de Candidatura ao Grau de Doutor em Farmacologia e Toxicologia Experimentais e Clínicas, submetida a Faculdade de Medicina da Universidade do Porto

Orientador – Professor Doutor Paulo Jorge da Silva Correia-de-Sá (MD, PhD)
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“Intelligence is the ability to adapt to change.”

Stephen Hawking

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Abbreviation list

$[Ca^{2+}]_i$ – intracellular calcium

A_1R – adenosine A_1 receptor

A_{2A}R – adenosine A_{2A} receptor

A_{2B}R – adenosine A_{2B} receptor

A_3R – adenosine A_3 receptor

AC – adenylyl cyclase

ADA – adenosine deaminase

ADO – adenosine

ADP – adenosine diphosphate

AMP – adenosine monophosphate

ATP – adenosine 5'-triphosphate

BCA- bicinchoninic acid

cAMP – cyclic adenosine monophosphate

CBX – carbenoxolone

Cx – connexin

Cx43- connexin43

CD73- Ecto-5'-nucleotidase

CGS 21680- 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2 yl]amino]ethyl]benzenepropanoic acid hydrochloride

DMEM- dulbecco's modified eagle medium

DMSO- dimethylsulphoxide

ECM- extracelular matrix

Ecto-5'-NT – ecto-5'-nucleotidase

EDTA- ethylenediaminetetraacetic acid

EGTA – ethylene-bis(oxyethylenenitrilo)tetraacetic acid

E-NPP- ecto-nucleotide pyrophosphatase/phosphodiesteras

E-NTPDase/NTPDase – ecto-nucleoside triphosphate diphosphohydrolase

ENT- equilibrative nucleoside transporters

EPAC- exchange protein activated by cAMP

ESI-09- α-[2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-b-oxo-3-isoxazolepropanenitrile

FBS- fetal bovine serum

GJ- gap- junctions

HC- hemichannels

Hist- histamine

HPLC- high-performance liquid chromatography

HSCF- human subcutaneous fibroblasts

FFA – flufenamic acid

FRS- fascia research society

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GPCR – G-protein-coupled receptor

MTT- 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

MPS- myofascial pain syndrome

MSK- musculoskeletal pain

NaCl- sodium chloride

NECA- 5′-(N-ethylcarboxamide) adenosine, (2S,3S,4R,5R)-5-(6-aminopurin-9-yl)-N-ethyl-3,4-dihydroxyoxolane-2-carboxamide

PBS- phosphate buffered saline

PFA- paraformaldehyde

Panx – pannexin

Panx-1- pannexin-1

PBN- probenecid

Pmol- picomole

PKA – protein kinase A

TBS- tris-buffered saline

SCH442416- [4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine

SD- standard deviations

SQ22,536- 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine

S.E.M.- standard error of the mean

$t_{1/2}$- half-life time

WB- western blot

Resumo

A dor musculoesquelética é a principal causa de dor crônica e está associada a um custo físico, emocional e financeiro considerável. Acredita-se que o tipo mais comum de dor musculoesquelética seja a síndrome dolorosa miofascial caracterizada pela presença de pontos de gatilho ao longo da fáscia superficial. Considerando que a fáscia é ricamente inervada, alterações na normal arquitetura desse tecido podem alterar a perceção da dor. Os fibroblastos são os principais constituintes da fáscia e são responsáveis por produzirem os principais componentes da matriz extracelular desse tecido. A demonstração recente da existência de uma rede ininterrupta de fibroblastos em todo o corpo realça a importância destas células no sistema fascial.Os fibroblastos são células muito dinâmicas que desempenham um papel ativo e muito mais complexo do que se pensava através da sua capacidade de reorganização interna do citoesqueleto, moderada atividade contráctil e influência sobre as propriedades viscoelásticas da matriz extracelular. Desta forma, a principal característica da fáscia é sua capacidade de adaptação, plasticidade e remodelação em resposta a vários níveis de stresse mecânico. O exagero do stresse físico (e mesmo emocional), ou a necessidade de remodelação contínua da fáscia devido a inflamação ou fibrose provoca a perda da sua elasticidade, tornando-se rígida, situação que se repercute de forma mais ou menos indefinida pelo resto do organismo.

As purinas, como a ATP e a adenosina, são moduladoras endógenas de múltiplas funções orgânicas. A sua libertação para o meio extracelular aumenta dramaticamente em resposta a situações de stresse ou à perda da energia celular, cuja origem pode ser muito diversa. O conhecimento das funções endógenas destes mediadores de stresse/homeostasia celular têm motivado um interesse crescente na procura de aplicações terapêuticas dirigidas à modificação local dos seus níveis e/ou funções. No caso da adenosina (foco principal desta dissertação), o seu papel tecidular é normalmente duplo. A adenosina funciona numa, fase inicial, como sinal de alarme, alertando as células vizinhas da existência de uma lesão tecidular potencial no sentido de se providenciar uma resposta protetora (e.g. redução da atividade do tecido, aumento da irrigação,

regulação da resposta inflamatória). Já a acumulação persistente do nucleósido no tecido pode desencadear processos patológicos como a inflamação e dor crónicas, bem como fibrose e/ou atrasos na cicatrização.

A reação dos tecidos ao ATP e aos seus metabolitos (e.g. adenosina) é normalmente interdependente, pleiotrópica e complexa devido ao aparecimento de ciclos viciosos de amplificação das respostas celulares às várias purinas com atividade biológica. Neste contexto, o presente trabalho foi delineado para explorar o papel das purinas, designadamente da adenosina, em fibroblastos humanos considerando os seguintes pressupostos: [1] a adenosina é um potente modulador da inflamação e da reparação tecidular com semivida relativamente curta, mas cujos níveis séricos refletem muitas vezes o exagero da sua produção em locais de stresse crónico [2] as alterações na regulação da sinalização mediada pelo ATP e pelos seus metabolitos podem ser importantes na remodelação da fáscia humana e, por conseguinte, no desenvolvimento de fibrose, inflamação e dor miofascial, [3] a translocação de ATP para o meio extracelular em células não excitáveis, incluindo os fibroblastos, ocorre através de difusão facilitada envolvendo a presença de hemicanaiscontendo conexinas (Cx) e/ou panexinas (Panx), por processos cuja regulação é relativamente desconhecida,mas que podem estar envolvidos na progressão de fibrose, inflamação e dor em diversos tecidos. Para tal, procedeu-se ao isolamento e realização de culturas primárias de fibroblastos subcutâneos provenientes de amostras de pele humana, para avaliar [1] a cinética das vias enzimáticas responsáveis pelo catabolismo extracelular do ATP e formação de adenosina nestas células, [2] a expressão e o papel dos recetores de adenosina na proliferação e na produção de colagénio, [3] o envolvimento da adenosina na sinalização intracelular mediada pelo cálcio em resposta a mediadores inflamatórios (e.g. histamina), e [4] a influenciada adenosina através da ativação seletiva dos seus recetores na expressão membranar e libertação de ATP para o meio extracelular através de hemicanais contendo Cx e/ou Panx em fibroblastos subcutâneos humanos (HSCF).

Os resultados mostram que o AMP (30 µM) é rapidamente (t½ 3 ± 1 min) desfosforilado em adenosina pela enzima membranar CD73 / ecto-5'-nucleotidase em HSCF. A acumulação extracelular de adenosina (t½ 158

± 17 min) nas culturas reflete uma baixa atividade da desaminase da adenosina (ADA) nessas células. Os HSCFs expressam imunorreatividade contra os recetores A_{2A} e A_3, mas são desprovidos de recetores A_1 e A_{2B}. Mostra-se, ainda, que a adenosina gerada a partir da hidrólise extracelular de nucleótidos de adenina facilita a produção de colagénio por HSCF através da ativação de recetores do subtipo A_{2A} sensíveis ao bloqueio pelo SCH442416. Isto foi demonstrado porque tanto o AMP, como o agonista seletivo do recetor A_{2A}, CGS21680, favoreceram a produção de colagénio sem afetar o crescimento das células em cultura. O acoplamento destes recetores à adenilato ciclase (AC) / proteína de troca ativada pelo AMP cíclico (EPAC) foi demonstrado usando inibidores farmacológicos, SQ22536 e ESI-09, respetivamente. Por fim, este conjunto de resultados mostram que o produto da desaminação da adenosina pela ADA, inosina, mimetizou o efeito do agonista do recetor A_3 da adenosina, 2-Cl-IB-MECA, já que ambos inibiram tanto a proliferação como a produção de colagénio em HSCF. O envolvimento do recetor A_3 na ação anti-proliferativa da inosina e da 2-Cl-IB-MECA foi assegurado através do bloqueio deste efeito pelo antagonista seletivo, MRS1191.

Os resultados obtidos nesta dissertação mostram pela primeira vez que a adenosina através da ativação do seu recetor A_{2A} controla a expressão dos hemicanais contendo Panx-1 e Cx43 e, através desse efeito, a capacidade dos HSCFs libertarem ATP para o meio extracelular em resposta a estímulos inflamatórios, como a histamina. A exposição dos HSCFs aos agonistas dos recetores A_{2A} da adenosina, NECA e CGS21680, durante 7 dias aumentou ($p < 0,05$) a imunorreatividade contra a Panx-1, causando um efeito oposto sobre a expressão dos hemicanais contendo Cx43. O aumento da expressão da Panx-1 induzida pela NECA e pelo CGS21680C nos HSCFs traduziu-se numa maior libertação de ATP por estas células comparativamente com células não tratadas provenientes dos mesmos indivíduos. O envolvimento do recetor A_{2A} nestes fenómenos foi confirmado através do uso do antagonista seletivo A_{2A} seletivo, SCH442416, que logrou abolir a resposta da NECA e do CGS21680. Também o inibidor dos hemicanais contendo Panx-1, probenecida, foi capaz de prevenir o efeito facilitatório dos agonistas A_{2A} na libertação de ATP pelos HSCFs. O aumento da expressão de hemicanais contendo Panx-1 promovida pela ativação

dos recetores A_{2A} da adenosina contribuiu para sustentar a acumulação intracelular de $[Ca^{2+}]_i$ nos HSCFs induzida pela histamina, muito provavelmente devido à facilitação da libertação de ATP para o meio extracelular.

Em conclusão, a adenosina gerada a partir da hidrólise extracelular dos nucleótidos da adenina acumula-se no meio extracelular e favorece a produção de colagénio por fibroblastos subcutâneos humanos por intermédio da ativação de recetores A_{2A} acoplados à via de transdução de sinal AC / EPAC. Descreve-se também neste trabalho pela primeira vez que a ativação dos recetores A_{2A} pela adenosina aumenta a expressão dos hemicanais contendo Panx-1, deslocando em sentido oposto a expressão de Cx43, nestas células. A sobre-expressão de Panx-1 induzida pela adenosina nos HSCFs favorece a libertação de ATP em resposta a estímulos inflamatórios (e.g. histamina) gerando-se um ciclo vicioso em que a acumulação de ATP no meio extracelular promove a sinalização mediada pelo Ca^{2+} e, também, maior acumulação de adenosina, cujos efeitos contribuem para a sustentação da resposta inflamatória, fibrose / remodelação e dor miofascial. Curiosamente, a infiltração do tecido subcutâneo / fáscia por células inflamatórias, normalmente enriquecidas em ADA nas suas membranas, favorece a desaminação da adenosina acumulada no meio extracelular em inosina, cuja ação inibitória do crescimento celular e, consequentemente, da migração e da reparação (produção de colagénio) tecidular por via da ativação de recetores A_3 contribui para a perpetuação da disfunção miofascial.

Os resultados apresentados contribuem, assim, para desvendar alguns dos fenómenos relacionados com a sinalização purinérgica mediada pela adenosina associada aos fibroblastos do tecido celular subcutâneo e, por via da comunicação intercelular, a todo o sincício miofascial, cujo conhecimento será decisivo para trabalhos futuros explorando outros aspetos deste sistema, tais como: 1) Compreender o envolvimento destes mecanismos moleculares no desenvolvimento e progressão das síndromes miofasciais dolorosas, certamente implicando modelos multicelulares (com células inflamatórias e/ou neurónios sensitivos) e multidimensionais (e.g. cutluras 3D); 2) esclarecer a participação da sinalização purinérgica fascial na saúde

(resposta adaptativas ao trauma e stresse) e na doença (doenças inflamatórias locais e sistémicas, fibrose e síndromes hiperproliferativos, dor crónica miofascial), incluindo a possibilidade de desenvolver novos biomarcadores de diagnóstico e seguimento destas patologias; 3) avaliação de novas estratégias terapêuticas locais e/ou sistémicas com base no sistema purinérgico aproveitando a sua capacidade de modular rapidamente a comunicação entre células (fibroblastos, células inflamatórias e terminações nervosas sensoriais) em resposta a estímulos de natureza muito diversa.

Abstract

Musculoskeletal pain is the main cause of chronic pain and is associated with physical, emotional and financial costs. The most common type of musculoskeletal pain is the myofascial pain syndrome, which is characterized by the presence of trigger points along the superficial fascia. Considering that the fascia is richly innervated, alterations in the normal architecture of this tissue can alter the perception of pain. Fibroblasts are the main constituents of fascia and are responsible for producing components of the extracellular matrix. The recent demonstration of the existence of an unbroken network of fibroblasts throughout the body highlights the importance of these cells in the fascial system. Fibroblasts are dynamic cells that play an active and complex role through their capacity for internal cytoskeleton reorganization, moderate contractile activity and influence on the viscoelastic properties of the extracellular matrix. Thus, the main characteristic of fascia is its adaptability, plasticity and remodeling capacity in response to various levels of mechanical stress. Increase of physical and emotional stress, or the need for continuous remodeling of the fascia due to inflammation or fibrosis causes the loss of its elasticity, with unknown repercussions for the rest of the body.

Purines, like ATP and adenosine, are endogenous modulators of multiple organ functions. Its release to the extracellular environment dramatically increases in response to stress or to the loss of cellular energy. The knowledge of the endogenous functions of these mediators of stress/cellular homeostasis has motivated a growing interest in the search for therapeutic applications aimed at local modification of their levels and/or functions. In the case of adenosine (the main focus of this dissertation), its tissue roles progresses in two distinct phases. At early stages, adenosine acts as an alarm signal, alerting neighboring cells to the existence of potential tissue damage in order to provide protective reactions (e.g. reduced tissue activity, increased irrigation, regulation of the inflammatory response). Persistent accumulation of the nucleoside in the tissue, can trigger pathological processes, such as chronic inflammation and pain, as well as fibrosis.

The tissue reaction to ATP and its metabolites (including adenosine) is normally interdependent, pleiotropic and complex due to the appearance of vicious cycles of amplification of the cellular responses to the various biologically active purines. In this context, the present work was designed to explore the role of purines, namely adenosine, in human fibroblasts considering the following assumptions: [1] adenosine is a potent modulator of inflammation and tissue repair, which levels in the serum may be indicative of the amounts of the nucleoside produced at chronic stress locations (e.g. fibromyalgia), [2] changes in the regulation of signaling mediated by ATP and its metabolites may be important in the remodeling of the human fascia and, therefore, in development of fibrosis, inflammation and myofascial pain, [3] the translocation of ATP to the extracellular medium in non-excitable cells, including fibroblasts, occurs through facilitated diffusion involving the presence of hemichannels containing connexins (Cx) and/or pannexins (Panx), which may be involved in the progression of fibrosis, inflammation and pain. To this end, we proceeded to the isolation and culture of fibroblasts of the human subcutaneous tissue, to evaluate [1] the kinetics of the enzymatic pathways responsible for the extracellular catabolism of ATP and formation of adenosine, [2] the expression and role of adenosine receptors in collagen proliferation and production, [3] the involvement of adenosine in calcium-mediated intracellular signaling secondary to inflammatory mediators (e.g. histamine), and [4] the influence of adenosine through selective activation of its receptors on membrane expression and ATP release through hemichannels containing Cx and/or Panx in human subcutaneous fibroblasts (HSCF).

Results show that AMP (30 μM) is rapidly (t½ 3 ± 1 min) dephosphorylated to adenosine by CD73 / ecto-5'-nucleotidase in HSCF. The extracellular accumulation of adenosine (t½ 158 ± 17 min) reflects a low adenosine deaminase (ADA) activity in these cells. HSCFs express immunoreactivity against A_{2A} and A_3 receptors, but are devoid of A_1 and A_{2B} receptors. Adenosine generated from the extracellular hydrolysis of adenine nucleotides facilitates collagen production by HSCF through the activation of A_{2A} subtype receptors sensitive to blockade by SCH442416. Both AMP and the selective A_{2A} receptor agonist, CGS21680, favored collagen production without affecting cells growth. The coupling of these receptors to adenylate cyclase

(AC) / cyclic AMP activated exchange protein (EPAC) was demonstrated using pharmacological inhibitors, SQ22536 and ESI-09, respectively. Finally, these results show that the product of adenosine deamination by ADA, inosine, mimicked the effect of the adenosine A_3 receptor agonist, 2-Cl-IB-MECA, and inhibited proliferation and production of collagen in HSCF. The involvement of the A_3 receptor in the anti-proliferative action of inosine and 2-Cl-IB-MECA was confirmed by blocking this effect with the selective antagonist, MRS1191.

The results obtained in this dissertation show for the first time that adenosine, through the activation of its A_{2A} receptor, controls the expression of hemichannels containing Panx-1 and Cx43 and, through this effect, the ability of HSCFs to release ATP into the extracellular medium in response to inflammatory drugs, such as histamine. Exposure of HSCFs to adenosine A_{2A} receptor agonists, NECA and CGS21680, for 7 days, increased ($p<0.05$) immunoreactivity against Panx-1, causing an opposite effect on the expression of Cx43-containing hemichannels. Overexpression of Panx-1 caused by NECA and CGS21680C in HSCFs translated into greater release of ATP compared to untreated cells from the same individuals. The involvement of the A_{2A} receptor in these phenomena was confirmed using the selective A_{2A} antagonist, SCH442416, which abolished NECA and CGS21680 responses. The inhibitor of Panx-1 containing hemichannels, probenecid, was also able to prevent the facilitatory effect of A_{2A} agonists on ATP release by HSCFs. The A_{2A} receptor-mediated Panx-1 overexpression contribute to sustain intracellular $[Ca^{2+}]_i$ accumulation in HSCFs challenged by histamine, most likely due to the facilitation of ATP release into the extracellular medium.

In conclusion, adenosine generated from the hydrolysis of released adenine nucleotides accumulates in the extracellular environment and favors collagen production by human subcutaneous fibroblasts through the activation of A_{2A} receptors coupled to the AC/EPAC signal transduction pathway. We describe here, for the first time, that the activation of A_{2A} receptors by adenosine increases the expression of hemichannels containing Panx-1, shifting the expression of Cx43 in the opposite direction. Panx-1 overexpression induced by adenosine in HSCFs favors the release of ATP in response to inflammatory stimuli (e.g. histamine)

generating a vicious cycle in which the accumulation of ATP in the extracellular environment favors Ca^{2+} signaling and greater accumulation of adenosine, which effects contribute to the support of the inflammatory response, fibrosis / remodeling and myofascial pain. Interestingly, infiltration of the subcutaneous tissue/fascia by inflammatory cells that are normally enriched plasma membrane-bound ADA favors adenosine deamination into inosine, which inhibits cell growth and repair via A_3 receptors activation, thus contributing to prolong myofascial dysfunction.

These results contribute to unravel some of the phenomena related to purinergic signaling operated by adenosine in fibroblasts of the human subcutaneous tissue, which through intercellular communication may affect the entire myofascial syncytia. This knowledge will be paramount for future work exploring others aspects of this system, such as: 1) understanding the involvement of these molecular mechanisms in the development and progression of painful myofascial syndromes, using multicellular (with inflammatory cells and/or sensory neurons) and multidimensional models (e.g. 3D cultures); 2) clarify the participation of fascial purinergic signaling in health (adaptive response to trauma and stress) and in disease (local and systemic inflammatory diseases, fibrosis and hyperproliferative syndromes, chronic myofascial pain), including the possibility of developing new diagnostic and follow-up biomarkers of these pathologies; 3) evaluation of new local and/or systemic therapeutic strategies based on the purinergic system, making advantage of its ability to rapidly modulate communication between cells (fibroblasts, inflammatory cells and sensory nerve endings) in response to different stimuli.

CHAPTER 1. INTRODUCTION

1.1 FASCIA

Fascia Definitions

> *"Fascia is what holds us together. It's a net that suspends your organs, a high-tech adhesive that holds your cells in place while relaying messages between them. The network is so extensive and ubiquitous that if you were to lose every organ, muscle, bone, nerve and blood vessel, your body would still retain its shape." (Dr. Hélène Langevin)*

The classification and nomenclature of fascia is a topic of current debate in the scientific community (Bordoni, Escher, et al. 2021). Fascia is not just connective tissue but is a more complex and vital structure (Adstrum et al. 2017b). The morphologic definition is presented in Gray's Anatomy defining fascia as "a term applied to masses of connective tissue large enough to be visible to the unaided eye". Its structure is highly variable but, in general, collagen fibers in fascia tend to be interwoven and seldom show the compact, parallel orientation seen in tendons and aponeuroses (Standring 2008). The Federative Committee on Anatomical Terminology (FCAT) introduced the term "fascia superficialis" and "fascia profunda." The superficial fascia is a "whole loose layer of subcutaneous tissue lying superficial to the denser layer of fascia profunda." The deep fascia, according to this definition, lies below the superficial fascia. To Dorland's Illustrated Medical Dictionary "fascia is a sheet or band of fibrous tissue such as lies deep to the skin or forms an investment for muscles and various other organs of the body" (Anderson 2012). Finally, in the Stedman's Medical Dictionary we can find the following definition: "Fascia is a sheet of fibrous tissue that envelops the body beneath the skin; it also encloses muscles and groups of muscles and separates their several layers or groups" (Adstrum et al. 2017b).

Only recently, through the "Delphi Method", the terms *Fascia* and *Fascial System* have been properly characterized by the Fascia Research Society (FRS)(Stecco et al. 2016; Adstrum et al. 2017b). The *fascial*

system consists of the three-dimensional continuum of soft, collagen containing, loose and dense fibrous connective tissues that permeate the body. It incorporates elements such as adipose tissue, adventitia and neurovascular sheaths, aponeuroses, deep and superficial fasciae, epineurium, joint capsules, ligaments, membranes, meninges, myofascial expansions, periostea, retinacula, septa, tendons, visceral fasciae, and all the intramuscular and intermuscular connective tissues including endo-/peri-/epimysium. The fascial system interpenetrates and surrounds all organs, muscles, bones and nerve fibers, endowing the body with a functional structure, and providing an environment that enables all body systems to operate in an integrated manner (Adstrum et al. 2017b). On the other hand, *fascia* is a sheath or any other dissectible aggregations of connective tissue that forms beneath the skin to attach, enclose, and separate muscles and other internal organs (Adstrum et al. 2017b). Despite the scientific uncertainty, medical texts agree that fascia covers all structures of the body, creating a structural continuity that gives form and function to all tissues and organs. Fascial tissue has a ubiquitous distribution in the body system; it is capable of involving, interpenetrating, supporting and forming the bloodstream, bone tissue, meningeal tissue, skeletal organs and muscles (Bordoni et al. 2020). Thus, the widely used term continuum is congruent with the continuity of fascial tissue and has long been observed and described by several anatomists (Adstrum et al. 2017b).

Histological Composition of Fascia

Fascia is mainly composed of "loose and dense fibrous connective tissues, containing collagen, which permeate throughout the body". This part of the definition is based on the established histological recognition of connective tissue as a type of basic tissue that is taxonomically subdivided into three categories - embryonic connective tissue (mesenchymal, mucous connective tissue), connective tissue itself (dense and loose connective tissue) and specialized connective tissue (blood, bone, cartilage, adipose tissue,

hemopoietic tissue, lymphatic tissue)(FIPAT 2011). From this classification perspective, fascia is generally considered to be a form of connective tissue itself (Adstrum et al. 2017b).

Fascia is made up of cells and extracellular matrix (ECM). In general, the extracellular matrix is composed mainly of glycoproteins and proteoglycans, many of which are able to bind to specific sites on other glycoproteins, so that the matrix becomes a highly cross-linked gel. Collagen, a triple helix glycoprotein, is the key structural fiber that gives the fascia its ability to withstand stress (Kumka et al. 2012). There are twenty-eight types of collagen described (Gordon et al. 2010). Although type I collagen represents about 90% of the human body's collagen, fascia contains a number of collagen-type combinations including, but not limited, to types I, III, IV, V, VI, XII, XIV, XXI (Gelse et al. 2003). A combination of multiple types of collagen within the extracellular matrix forms a unique structure, like a design that reflects the function and conformity of the fascia in various regions (Kumka et al. 2012). The distribution and composition of extracellular matrix among tissues play important roles in the etiology, pathology and mechanism of diseases. At the cytological level, fascia includes fibroblasts, myofibroblasts, adipocytes and several migratory white blood cells. Fibroblasts are chief cells in the fascial system. Resident cells are responsible for the synthesis and maintenance of ECM, yet the extracellular matrix also significantly influences cellular functions (Gelse et al. 2003; Langevin, Nedergaard, et al. 2013). Fibroblasts indirectly influence matrix stiffness through the production and degradation of matrix proteins (Langevin, Bouffard, et al. 2011). During wound healing, fibroblasts can differentiate into myofibroblasts, which are able to contract and, thereby, generate measurable increases in connective tissue tension for long time periods (Langevin, Bouffard, et al. 2011).

Mechanical Functions of the Fascial System

Fascial system properties can be conceptually divided into microlevel (molecular and cellular responses) and macrolevel (mechanical) properties. The extracellular matrix (ECM) contains both molecular and cellular components. The cellular cytoskeleton is connected to the ECM through a molecular chain; this chain allows molecular interactions to occur in a dynamic, bidirectional fashion. Thus, functional and structural changes in the ECM can result in cellular adaptations and vice versa. Additionally, the ECM can serve as a molecular storage system, housing, and releasing biologically active molecules involved in organ or tissue regulation. The emanation or activation of these molecules can be triggered by mechanical stress (Ganjaei et al. 2020). One of the fundamental characteristics of fascia is its ability to adapt to mechanical stress and cellular remodeling, thus mirroring the functional needs of tissue microenvironments (Bordoni et al. 2018). The normal movement of the body is allowed by the presence of fascial tissues and their inseparable interconnection, which consents the sliding of the muscular structure, nerves and vessels between contractile structures and joints, and the ability of all organs to slide and move over each other according to the position of the body (Bordoni, Mahabadi, et al. 2021). Fibroblasts adapt their function according to the metabolic and mechanical stimuli these cells are submitted to; intercellular junctions between these cells help long distance cellular communication, thus making information available in real time to the entire body (Bordoni et al. 2015). If requirements change, for instance in terms of increased mechanical stress or prolonged immobilization, transcription of pro-collagen deoxyribonucleic acid (DNA) will change the types of collagen (e.g. from type I to type III) produced by fibroblasts, or even recruitment of undifferentiated cells to more differentiated cell lineages (Kumka et al. 2012).

In this way, fascia allows a more adequate distribution of the tension information amongst different tissues, so that the entire body can react in real time to both internal and external stimuli from the epidermis to more deeper organ systems and vice versa (Bordoni, Mahabadi, et al. 2021). The mechanical characteristics of the tissue may be directly influenced by fibroblasts, given their ability to control the composition of the

extracellular matrix. The number of molecular elements making up the extracellular matrix varies depending on the tissue tension. This relationship can be harmful when excessive tension is verified leading to fibrosis development; on the other hand, fascial morphological features and function attributes may be kept fairly conserved due to the plasticity of this tissue to correct for mechanical stress by changing its viscosity and elasticity (Bordoni, Mahabadi, et al. 2021)

Thus, overall, fascia can influence health conditions of individuals, as it is able to change the mechanometabolic environment, pain perception, inflammatory responses. A chronic deformation of the myofascial system causes mechanoceptors to become nociceptors, as occurring for instance in the thoracolumbar fascia which may simulate an idiopathic low back pain syndrome. The reduction in fascial tissue sliding may cause local inflammation underlying several neck pain conditions. Altered position of the collagen fibers within a tendon may change its mechanical characteristics, thus creating pain without any signs of inflammation. This may occur whenever the orientation of the various fascial layers lose their ability to move between each other, resulting in vectorial changes of collagen fibrils, altered collagen deposition and extracellular matrix composition, which may create an adequate environment for mechanical tension abnormalities and inflammation of underlying skeletal cells.

The myofascial system can also be closely related to emotional processing in painful conditions (Langevin 2008). In fact, the myofascial system has a very thin, wide, diverse and always present network innervation formed by myelinated proprioceptive endings (Ruffini, Golgi and Pacini), which are located inside the connective tissue or closely related to underlying muscles, periosteum, endomysium and perimysium, and visceral organs. Most often, these receptors convey proprioception, nociception and interoception (Bordoni, Mahabadi, et al. 2021). Interoception is defined as the sense of the physiological condition of the body, i.e. conscious awareness, emotional processing and behavior related to afferent physiological information arising from all over the body (Schleip et al. 2012). Thus, interoception might have an important role in the processing and regulation of chronic pain at the Central Nervous System level (Murphy et al. 2017; Chen

et al. 2021). This is a brand new area of investigation to understand chronic painful myofascial conditions, especially fibromyalgia (Borg et al. 2018).

1.2 ROLE OF FIBROBLASTS IN DEVELOPMENT OF MYOFASCIAL PAIN SYNDROME

Myofascial pain syndromes

Musculoskeletal pain has a major impact on people's quality of life and is associated with a high physical, emotional and financial cost (WHO 2021). Approximately 1.71 billion people have musculoskeletal problems worldwide. Musculoskeletal conditions significantly limit mobility and dexterity, leading to early retirement from work, lower levels of well-being and reduced ability to participate in society (WHO 2021). Due to population growth and aging, the number of people with musculoskeletal disorders is increasing rapidly. The most prevalent musculoskeletal pain conditions are low back pain and myofascial syndromes, such as fibromyalgia (Hawker 2017).

The myofascial pain syndrome includes sensory, motor, and autonomic symptoms such as decreased range of motion and, consequently, decreased quality of life. Although the myofascial pain syndrome is complex in its presentation, it is known that its onset and persistence is caused by myofascial trigger points (Jafri 2014). In patients, myofascial trigger points appear as focal areas in the muscle that appear stiff, hypercontracted and are painful when finger pressed. Despite the causal association of myofascial trigger points with the underlying physiology of the myofascial pain syndrome, the mechanisms that cause the initiation and maintenance of myofascial trigger points are unknown. Only through a better molecular understanding of the subcellular pathways behind this disorder can new therapeutic options be uncovered. Better understanding of these phenomena may also help to propose and/or optimize treatment options. However, many gaps in our knowledge exist regarding these signaling pathways deserving further investigations (Jafri 2014).

Myofascial pain syndromes can have different etiologies, including direct or indirect trauma, pathological exposure to cumulative and repetitive tension, postural dysfunction, hypomobility and intense physical

exercise, among other more or less specific disease conditions. Thus, treatment of the underlying cause is currently the most widely accepted strategy for myofascial pain syndromes therapy. The lack of diagnostic criteria for myofascial pain syndromes undermines timely and correct diagnosis, and, therefore, proper treatment. Current pharmacological approaches include oral nonsteroidal anti-inflammatory drugs (NSAIDs) as they are readily available and have a relatively mild side-effect profile. However, long-term use should be considered with caution due to the gastrointestinal, renal, and antiplatelet side effects (Desai et al. 2013). Selective cyclooxygenase-2 inhibitors (Coxibs) are about equipotent to traditional NSAIDs with relatively more tolerable side effects. Nevertheless, these new compounds should not be considered in patients with a history of thrombosis and cardiovascular events. Muscle relaxants are a group of drugs with varying pharmacology that act on the central nervous system to disrupt nociceptive pain (Borg-Stein et al. 2014). Cyclobenzaprine targets muscle relaxation without affecting muscle function. Its mechanism of action is not known, but its structure is similar to that of tricyclic antidepressants. It is often used for both pain relief and sleep induction, because it has a sedating effect. In 41 patients with myofascial jaw pain, cyclobenzaprine was slightly better than clonazepam or placebo in pain relief, yet it was not more effective at ameliorating sleep (Borg-Stein et al. 2014). Tropisetron, a 5-HT3 receptor antagonist and an α7-nicotinic receptor agonist, has been recently introduced as analgesic for fibromyalgia and myofascial pain conditions with a limited commercial availability (Müller et al. 2004). Anticonvulsants, like gabapentin and pregabalin, display analgesic, anxiolytic-like, and anticonvulsant activity by reducing the release of several neurotrasnmitters involved in nociception, including glutamate, noradrenaline and substance P. Tricyclic antidepressants (TCAs) are indicated for chronic pain, fibromyalgia and neuropathic pain. Although its mitigating effects on pain perception are not fully understood, it is postulated that TCAs act by favoring the central serotonergic and noradrenergic descendent inhibitory signals that control pain perception (Desai et al. 2013). Other treatments, such as botulinum toxin type A (BoNT-A) that acts to prevent muscle

contraction. have been investigated and may have analgesic properties by decreasing the production of substance P (Fallah et al. 2012).

Among the non-pharmacological treatments currently described, dry needling has been traditionally used as one of the fastest and most-effective ways to inactivate the trigger points and help alleviate the accompanied pain. Trigger point injections consists in the administration of various solutions, typically local anesthetics. Studies suggest similar efficacy to dry needling but with less discomfort (Ay et al. 2009). A systematic review article on needling therapies for trigger points found that, based on current medical evidence, the ‘‘nature of the injected substance makes no difference to the outcome and that wet needling is not therapeutically superior to dry needling’’ (Cummings et al. 2001). In addition, manual therapy, including deep pressure massage and myofascial release are commonly used for the treatment of myofascial pain syndromes, since they are considered to be the most effective techniques for inactivating trigger points. Other techniques including the use of ultrasound, laser therapy, magnetic stimulation are described in the literature and reviewed in clinical trials, however their effectiveness needs further investigation (Ay et al. 2009).

However, most interventions, whether pharmacological or non-pharmacological, demonstrate limited scientific evidence for their use. This shortage of high-quality evidence is probably due to the complex pathology of myofascial pain syndromes comprising both central and peripheral neural mechanisms. Therefore, we predict that the investigation of the molecular mechanisms underlying the chief signaling role of fascial fibroblasts may contribute to unveil modifications in the entire body system caused by myofascial syndromes, such as fibromyalgia.

Myofascial pain syndromes: The central role of fibroblasts

The main characteristic of the connective tissue is its plasticity in response to various levels of mechanical stress (Langevin 2008). Local chronic increases in stress can lead to micro-injuries and inflammation, while a consistent absence of stress leads to connective tissue atrophy, architectural disorganization, fibrosis, adhesions and contractures. Altered tension due to the disorganization of richly innervated tissue structures can modify sensory inputs from the body affecting tissues, making sensory neurons more responsive (Langevin 2014a). Furthermore, a sustained disturbance of non-specialized connective tissue can induce a pathological neuroplastic rearrangement of its sensory innervation, thus contributing to the development of peripheral sensitization followed by central sensitization. The viscoelastic properties of fascial connective tissue (e.g., stiffness, cushioning) are determined by its architecture, molecular composition (mostly collagen, elastin, and glycosaminoglycans), and water content, all of which are mutually interdependent.

Thus, the fascia appears to play an important role in the development and progression of chronic painful myofascial diseases. Several studies have shown that chronic inflammation and connective tissue fibrosis may play a role in chronic musculoskeletal pain (Langevin, Fox, et al. 2011b). Individuals with chronic low back pain have increased thickness and decreased mobility of connective tissue in the back. Fibrosis of the connective tissue due to injury or chronic inflammation causes restricted mobility, decreased shear stress, and decreased stretching of loose connective tissue during body movements. In the presence of fibrosis, the lack of fibroblast remodeling in response to tissue stretching could further stiffen the tissue and contribute to spread of the pathology to adjacent areas (Langevin 2014a). In addition, *in vivo* and *ex vivo* experiments have shown that stretching connective tissue for 10 minutes has antifibrotic effects (Abbott et al. 2013).

A new model for better understanding the pathophysiology of chronic musculoskeletal pain, incorporating connective tissue remodeling and neuroplasticity, was recently presented (see Figure 1). Connective tissue is richly innervated with mechano-sensory and nociceptive neurons. Modulation of nociceptor activity has been shown to occur in response to changes in the innervated tissue. Although it is unclear whether

myofascial pain trigger points are the cause or consequence of muscle injury, they represent abnormally contracted muscle fibers, which can lead to the accumulation of signaling molecules that are essential for the initiation and maintenance of inflammatory reactions, such as prostaglandins, bradykinin, histamine, ATP and serotonin, among others. Some of the signaling substances that accumulate in such microenvironment can activate nociceptors. Thus, inflammation can be directly involved in pain as well, due to activation/modulation of nociceptive substances activity/neurotransmission threshold induced by inflammatory mediators. On the other hand, the constitutive activation of nociceptors can contribute to the development or worsening of fibrosis and inflammation, causing even more tissue stiffness and pain escalation.

In the skin, it has been shown that the release of substance P by sensory C fibers can increase the production of histamine and cytokines from mast cells, monocytes and endothelial cells. Furthermore, increased production of transforming growth factor β1 (TGFβ-1), stimulated by tissue injury and histamine release, seems to be a powerful booster of fibroblast collagen synthesis and tissue fibrosis. Thus, a better understanding of fascial multicellular signaling and tissue remodeling mechanisms is paramount to propose new therapeutic strategies for myofascial pain syndrome (Langevin, Nedergaard, et al. 2013).

Fibroblasts are the main cellular constituent of fascia. Increasing evidence demonstrate that fibroblasts from a continuum network throughout the body emphasizing the chief role of these cells in fascial communication. The biomechanical behavior of connective tissue in response to stretching is generally attributed to the molecular composition and organization of its extracellular matrix. In this endeavor, fibroblasts also play active roles in regulating connective tissue tension. Each fibroblast is potentially aware of the functional state of both neighboring cells and those that are far from it, thus ensuring fascial and mechanical continuity (Langevin et al. 2004). In response to static tissue stretching, fibroblasts expand within minutes by active remodeling of their cytoskeleton (Langevin, Nedergaard, et al. 2013). Fibroblasts secrete the main components of the fascial extracellular matrix (collagen, glycosaminoglycans (GAGs) and

elastin) and, therefore, play a fundamental role in determining its composition. Water content, for example, is dependent on both the concentration of negatively charged glycosaminoglycans (GAGs) that bind large amounts of water and on the organization, strength, and stiffness of the collagen fiber network, which resists tissue swelling (Langevin 2008).

Mechanical stimuli applied to the entire tissue, such as stretching, cause direct local mechanical deformation of the cytoskeleton of these cells. When connective tissue is stretched, fibroblasts respond within minutes by expanding, flattening and actively reorganizing their cytoskeleton (Langevin et al. 2005) . In addition, guiding various types of cells, such as mast cells, T cells, B cells, among others cells, through the release of signaling molecules, fibroblasts facilitate their recruitment, retention, activation and differentiation (Davidson et al. 2021).

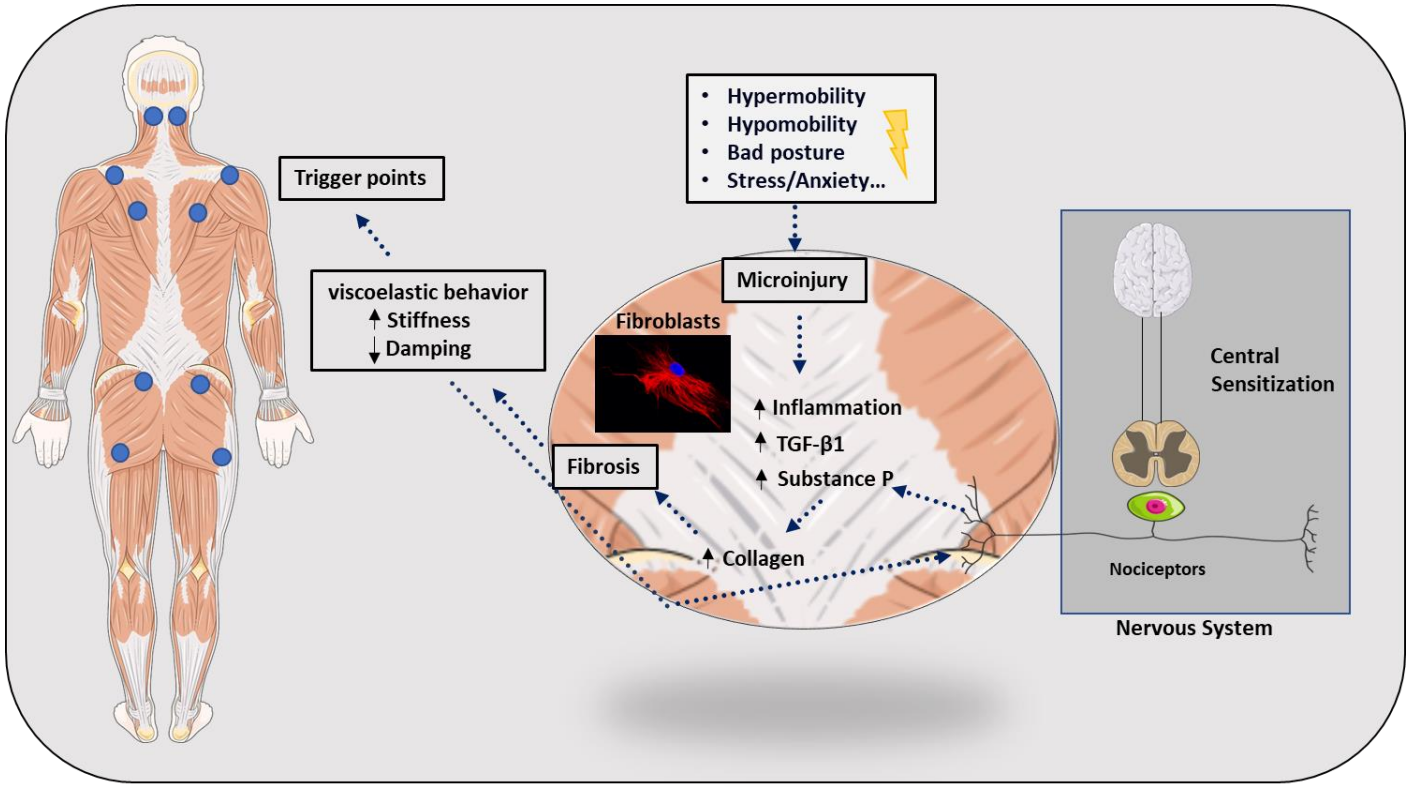


Figure 1: Fascia in Myofascial pain. Relationship between Fascia plasticity and Neuroplasticity. Figure adapted from (Langevin 2008)

1.3 THE PURINERGIC SYSTEM

Purinergic signaling

Adenine nucleotides, like ADP and ATP and nucleosides (e.g. adenosine) are widely known for their crucial role in DNA/RNA synthesis and energy metabolism (Rang 2020). The existence of a purinergic communication system in which ATP plays a chief role was first coined in 1972 by Geoff Burnstock (Burnstock et al. 1972). Although this theory was initially discredited by the scientific community, later on it was shown that ATP is released by nerves supplying the bowel and bladder (Burnstock 1972, 2014), as well as from many other neuronal and non-neuronal cells throughout the body (Burnstock 2018c). It signaling actions are mediated by plasma membrane-bound receptors, which may also sense extracellular ATP metabolites, like ADP, AMP and adenosine. Purinoceptors activation trigger a variety of intracellular signaling cascades, thus providing strict control over intra and intercellular communication in various tissues (Burnstock 2018c, 2018b). Depending on the purinoceptor subtype being activated, ATP may exert short-term effects most likely as a neurotransmitter or neuromodulator, while also playing significant roles in long-term (trophic) phenomena affecting cells proliferation, differentiation and death (Burnstock 2018b). Nowadays, there is growing evidence that the purinergic signaling cascade is altered in many pathological conditions, including stroke and thrombosis, osteoporosis, pain, chronic cough, kidney failure, urinary incontinence, cystic fibrosis, cancer and disorders of the CNS (e.g. Alzheimer's, Parkinson's, multiple sclerosis), which has open new avenues for therapeutic interventions directed towards normalization of this important signaling pathway (Lopes et al. 2021; Burnstock 2018c; Borea et al. 2016).

Nucleoside and nucleotide-releasing pathways

For many years, the main source of extracellular nucleosides and nucleotides was considered damaged or dying cells, except in cells (like neurons) where vesicle exocytosis is highly probable. This concept has now been changed given to the discovery that many cell types constitutively release in response to both physiological and pathological stimuli. Thus, in addition to vesicle exocytosis, non-vesicular mechanisms can convey the release of ATP across the plasma membrane (Burnstock 1999; Romanello et al. 2005; Knight et al. 2002). Transportation of highly charged ATP molecules across the plasma membrane requires large anion-conductance pores. Nowadays, five groups of channels were recognized by their ability to release ATP: the calcium homeostasis modulator 1 (CALHM1), volume-regulated anion channels (VRACs, also known as volume-sensitive external rectifying anion channels - VSOR), maxi-anion channels (MACs) and hemichannels containing connexins and pannexins. Previously, our group demonstrated that subcutaneous human fibroblasts express Panx-1 and Cx43 hemichannels and play an important role in signaling these cells and fascia remodeling (Pinheiro et al.; 2013a; Pinheiro et al., 2013b). Moreover, changes in the activity and expression of connexin43 (Cx43) and pannexin-1 (Panx-1) hemichannels have been implicated in numerous physiological and pathological conditions including fibrosis, inflammation and chronic pain.

ATP-releasing hemichannels containing connexins and pannexins

Hemichannels (HC) containing either connexins and pannexins provide communication between the cytosol and the extracellular environment of individual cells, even though autocrine and paracrine phenomena by released molecules might also occur, while gap junctions (GJ) containing connexins support the exchange of a series of molecules between the cytoplasm of adjacent cells (Willebrords et al. 2017). Under physiological conditions, GJs are usually opened, while hemichannels containing connexins and pannexins constitute selective pores, opening or closing depending on the physiological or pathological conditions,

which include oxidative and mechanical stresses, decreases and/or increases in the calcium concentration gradients across the plasma membrane (Willebrords et al. 2017).

Connexins were first described in the early 1970's as major proteins contained in intercellular GJs. Currently, 21 members of the connexin family have been identified in humans. All connexin subtypes share the same topology consisting of 4 transmembrane domains (M1-M4), 2 extracellular loops (E1-E2) and 1 N- and C-terminal cytoplasmic loops (Gilula 1996). The pannexin family was identified more recently and comprises 3 members, namely Panx1, Panx2 and Panx3. They assemble in connexin-like configurations, but do not form GJs due to extensive glycosylation in the second extracellular loop (Wang et al. 2013). Gap-junctions, like connexin-containing hemichannels, are well represented in human subcutaneous fibroblasts and play important roles in tissue signaling and remodeling.

Hemichannels are increasingly considered emerging targets in the treatment of various disease conditions, such as inflammation, fibrosis and pain (Chekeni et al. 2010; Laird et al. 2018; Mori et al. 2006; Morioka et al. 2019). Many different molecules are known to inhibit connexin- and pannexin-containing hemichannels; these may be grouped as chemical-based inhibitors, RNA-based inhibitors, antibody-based inhibitors and peptide-based inhibitors (Willebrords et al. 2017). Among the chemical-based inhibitors, one can mention the glycyrrhetinic acid and its derivatives (e.g. carbenoxolone), long-chain alcohols (e.g. octanol and heptanol), halogenated volatile anesthetics (e.g. halothane and enflurane), fatty acids (e.g. oleic acid and arachidonic acid), fenamates (e.g. flufenamic acid), quinine and analogs (like mefloquine), 2-aminoethoxydiphenyl borate (e.g. 2-APB), polyamines (e.g. spermine) and probenecid (Willebrords et al. 2017). Probenecid is used clinically for the treatment of gout and to increase effective concentrations of antibiotics and chemotherapeutics (Silverman et al. 2008b). The proposed mechanism of action is similar to carbenoxolone, showing some degree of selectivity to interact with the first extracellular loop of Panx1 (IC_{50} 150 µM) (Silverman et al. 2008b). Among chemically-based inhibitors, probenecid has been reused as a "specific" inhibitor of PANX1 channels to study the functional role of PANX1 in animal models. Thus,

probenecid is widely used in preclinical trials due to its greater selectivity to Panx-1 compared to Cxs hemichannels.

Despite the many efforts made in the last 2 decades to identify potent inhibitors of these cellular pores, most of these compounds show non-specific and/or non-selective characteristics as they inhibit several subtypes of connexin- and pannexin-containing hemichannels, as well as GJ communication (Laird et al. 2018). Synthetic peptides, in contrast to chemically-based inhibitors, have better selectivity for connexin- vs pannexin-containing hemichannels (Willebrords et al. 2017; Caufriez et al. 2020). For instance, the peptide mimetics, ^{10}Panx1 and PanxE1b, reproduce sequences of the first extracellular loop of Panx1, while IL2 mimics the cytoplasmic loop of Panx1. Both Panx305-318 hPanx(Ct) and hPanx1(Ct)Δ391 mimic sequences of the C-terminal of Panx1 (Caufriez et al. 2020). As for the connexin protein subtypes, Cx37, Cx40 and Cx43 are mimicked by peptides under the Gap26 code located in the first extracellular loop of connexin. Gap27 and Peptide5, on the other hand, mimic regions of the second extracellular loop of these proteins; Gap27 targets Cx32, Cx40 and Cx43, while Peptide5 is used to inhibit Cx43. JM2, ΔSH3, CT9, CT10, αCT mimic the C-terminal of Cx43. Both L2 and Gap19 also mimic the cytoplasmic loop from Cx43 (Caufriez et al. 2020).

Although a great deal of information has been collected about connexin- and pannexin-containing hemichannel inhibitors, little is known about the activators of these channels. Nevertheless, several peptides that putatively open these hemichannels have already entered clinical trials. Topical administration of ACT1 peptide, targeting Cx43, has been shown to increase healing of chronic neuropathic foot ulcers and venous leg ulcers in diabetic patients (Ghatnekar et al. 2015).

In general, peptides are highly potent signal transduction molecules that exert powerful physiological effects. However, these compounds are characterized by having a relatively short circulating plasma half-

life, as well as sub-optimal physical and chemical properties to be considered as therapeutic options (Willebrords et al. 2017; Fosgerau et al. 2015).

The ectonucleotidase cascade as a source of extracellular adenosine

The purinergic signaling is by far more complex than of any other known neurotransmitter / neuromodulator system, as its signal transduction mechanisms are strictly dependent on the extracellular levels of their ligands, which are ultimately modulated by the activity of a series of co-localized nucleotide-hydrolytic enzymes (Burnstock 2014). Once in the extracellular space, ATP can be dephosphorylated to produce other purine-derived molecules such as ADP, AMP and ADO by ecto-nucleotidases. Consequently, ATP may exert dual functions, being able to directly activate ATP-sensitive P2X and/or P2Y receptors, while upon its breakdown into ADP and ADO may further activate other P2Y subtypes, as well as adenosine-sensitiveP1 receptors, respectively. By controlling the duration of the ATP action on its receptors and the biochemical nature of its metabolites near plasma membrane receptors, ecto-nucleotidases seem to be crucial to fine-tune purinergic signaling (Zimmermann 2020).

Ecto-nucleotidase families include the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases, CD39), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP), alkaline phosphatases, and ecto-5'-nucleotidase (CD73). There are four members of the E-NTPDase family, namely E-NTPDase1, E-NTPDase2, E-NTPDase3 and E-NTPDase8 (Robson et al., 2006). These enzymes have distinct biochemical properties. E-NTPDase1 hydrolyzes ATP and ADP equally well, E-NTPDase2 preferentially hydrolyzes triphosphonucleosides, and E-NTPDase3 and E-NTPDase8 have an intermediate hydrolysis profile (Kukulski et al., 2005). The hydrolysis of tri- and diphosphonucleosides by E-NTPDases yields AMP as the final product, which can be fully dephosphorylated to adenosine by ecto-5'-nucleotidase (CD73) (Colgan et al., 2006). The relative contribution of the distinct ectonucleotidase species to the modulation of purinergic

signaling may depend on differential tissue and cell distribution, regulation of expression, targeting to specific29 membrane domains, but also on substrate availability and substrate preference. In human subcutaneous fibroblasts, results demonstrated that, once in the extracellular milieu, ATP (3/30 μM) and ADP (3/30 μM) were metabolized with a ratio of approximately 2(ATP):1(ADP), which is compatible with E-NTPDase1 being the most effective isoform of E-NTPDases in these cultures. Furthermore, human subcutaneous fibroblasts showed a stronger immunoreactivity to E-NTPDase1 when compared to E-NTPDase2, and these cells presented no immunoreactivity against NTPDase3 (Pinheiro et al. 2013a; Pinheiroet al. 2013b).

Extracellular ATP/ADP breakdown by ecto-nucleotidases (like CD39 and CD73) ultimately generates adenosine, which is found ubiquitously in all fluids throughout the body (Burnstock 2017b). Extracellular adenosine concentrations are generally quite low compared to the intracellular levels of the nucleoside. Extracellular adenosine acts through P1 receptors, often opposing the effects of P2 receptor signaling. Under healthy physiological conditions, the impact of ATP and adenosine signaling is balanced by ectonucleotidases that stabilize the autocrine and paracrine cellular environment. Within pathophysiological conditions, this homeostatic balance can be disturbed due to increased nucleotide release and dynamic changes in ectonucleotidase and receptor expression. The concept of the "CD39 adenosinergic axis" has been introduced to emphasize the important role of sequential ATP degradation and adenosine formation by NTPDase1 (CD39) and ecto-5′-nucleotidase (CD73), respectively in these configurations (Zimmermann 2020).

Adenosine is mainly produced from the metabolism of adenosine triphosphate (ATP) both intracellularly and extracellularly. Intracellular adenosine is produced of its immediate precursor, 5'-adenosine monophosphate (5'-AMP), by the action of the enzyme 5'-nucleotidase. Adenosine can then follow several metabolic/synthetic pathways. It might be metabolized to inosine and hypoxanthine by adenosine deaminase and to uric acid by xanthine oxidase (Latini et al. 2001). Adenosine can also be transported out of the cell

into the extracellular space by specific bi-directional nucleoside transporters. Adenosine transporters belong to two distinct gene families that encode plasma membrane-bound equilibrative (ENT) and concentrative (CNT) nucleoside transporter proteins, respectively (Pastor-Anglada et al. 2018). Inhibitors of these transporters, such as dipyridamole, increase the extracellular concentrations of adenosine and are clinically useful to treat certain complications. Adenosine can also be converted back to 5'-AMP by the action of the enzyme adenosine kinase and subsequently to ADP and ATP (Latini et al. 2001) (See Figure 2). Into the extracellular space, adenosine mediates its physiological effects through the activation of four ARs (A_1, A_{2A}, A_{2B} and A_3).

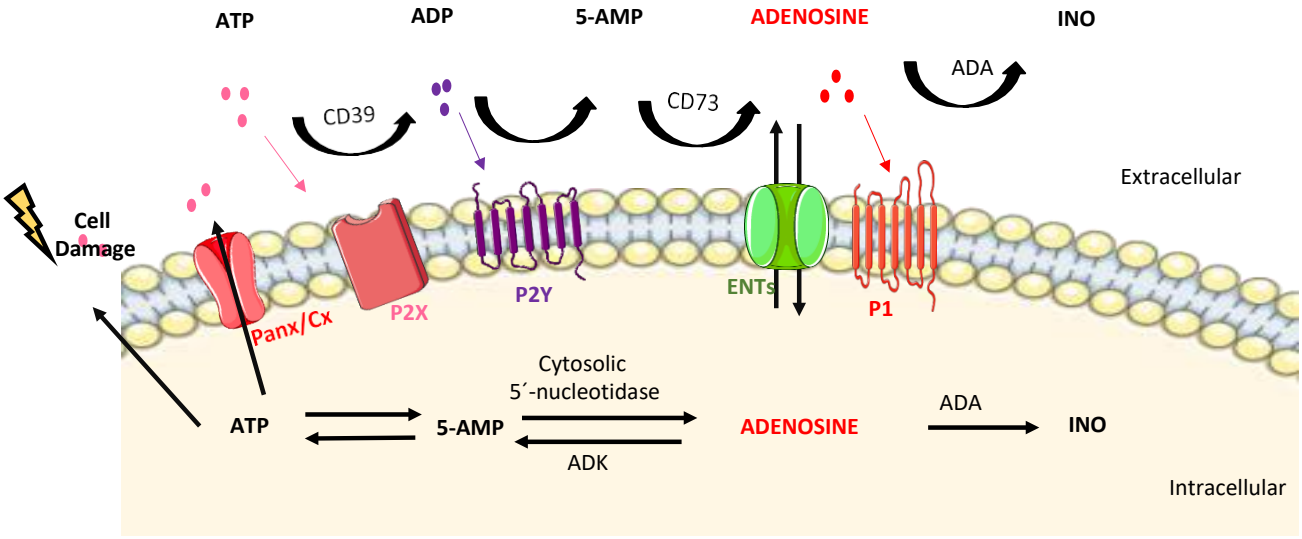


Figure 2: Adenosine metabolism and transport in the extra-intracellular milieu. Figure adapted from (Borea et al. 2018; Junger 2011; Huang et al. 2021)

Adenosine receptors

Adenosine acts through the activation of four distinct receptor subtypes: A_1, A_{2A}, A_{2B} and A_3. Adenosine receptors (ARs) belong to the superfamily of G-protein coupled receptors (GPCRs)(Burnstock 2018b, 2018c). All ARs possess seven transmembrane α-helical structure with an extracellular amino terminus and an intracellular carboxyl terminus; the N-terminal domain has N-glycosylation sites that influence trafficking of the receptor to the plasma membrane. In rodents, A_1 and A_{2A} ARs possess high affinity for adenosine, while the A_{2B} and A_3 ARs show relatively lower affinity for adenosine receptors (Burnstock 2018b); this scenario may change in humans where the A_3 AR also have high affinity for the nucleoside. A_1 and A_3 are negatively coupled to adenylate cyclase, while A_{2A} and A_{2B} are positively coupled (Burnstock 2018c).

The A_1AR subtype induces phospholipase C activation, thereby increasing inositol 1,4,5-trisphosphate (IP3) and intracellular Ca^2 levels, which stimulate calcium-dependent protein kinases (PKC) and/or other calcium-binding proteins. Furthermore, the involvement of A1AR in the intracellular phosphorylative cascade of the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK), p38, and Jun NH2-terminal kinase (JNK) has been reported (Borea et al. 2018).(Borea et al. 2018; Huang et al. 2021)The A_{2A}R signals via Gs signal transduction proteins which activate adenylate cyclase, increasing cytosolic cAMP. Elevated intracellular cAMP activates both protein kinase A (PKA) and Epac (Perez-Aso and Mediero 2013). A_{2B}AR signaling pathways involve AC activation through Gs proteins, leading to PKA phosphorylation and enrollment of different cAMP-dependent effectors like exchange proteins, which are directly activated by cAMP (Epac). In addition, A_{2B}ARs can stimulate PLC through the Gq protein, resulting in Ca^{2+} mobilization, and can regulate ion channels through their subunits. Moreover, this subtype acts as stimulator of MAPK activation in several cell models in both central and peripheral systems (Borea et al. 2016).A_3ARs trigger a variety of intracellular signaling by preferentially coupling to Gi proteins, by which they reduce cAMP levels, PKA inhibition, wich leads to an increase in

glycogen synthase kinase-3β (GSK-3β) and consequent decrease of beta-catenin, cyclin D1 and c-Myc. High concentrations of A_3AR agonists, to Gq proteins or Gβγ subunits, thereby induces an increase in both PLC and calcium. Phosphorylation of p38, ERK1 / 2 and JNK1 / 2 is also induced by activation of A_3AR (Borea et al. 2018; Borea et al. 2016; Huang et al. 2021).

Selective agonists and antagonists of the P1 subtype have been identified (see Table 1). These receptors are widely expressed and have been implicated in various physiological and pathological conditions (Fredholm et al. 2011). These include neuromodulation (including brain excitability and sleep regulation, control of immune responses, heart rhythm and the reactivity of blood vessels, lipolysis, angiogenesis, but also influence inflammation, pain sensation and neurodegeneration conditions (Fredholm et al. 2011; Jacobson et al. 2016).

Table 1: Adapted from: (Borea et al. 2018; Burnstock 2018a; Jacobson et al. 2016)

Adenosine Receptor	Human Gene	Adenosine affinity	G protein coupling	Transduction Mechanisms	Agonists	Antagonists
A_1	$ADORA_1$	1-10nM	Gi /Go	↓ Adenylyl cyclase ↓ cAMP	Adenosine,CCPA, R-PIA, CPA, IB-MECA, NECA,MRS5474, INO-8875	PSB36, N-0840, MRS1754, PSB 36, PQ-69, CGS16943, KW-3902, DPCPX, cafeine, theophyline
A_{2A}	$ADORA_2A$	30nM	Gs	↑ Adenylyl cyclase ↑cAMP	Adenosine, CGS21680, UK-432,097, HE-NECA, NECA, CV-1808, R-PIA, Regadenoson	SCH442416,KW 6002, ZM241385, SCH58261, DPCPX, cafeine, theophyline
A_{2B}	$ADORA_2B$	1000nM	Gs	↑ Adenylyl cyclase ↑ cAMP	Adenosine, NECA, BAY60-6583, R-PIA, NECA	PSB-603, MRE-2029-F20, GS-6201, ZM241385, MRS 1754, DPCPX, , cafeine, theophyline
A_3	$ADORA_3$	100nM	Gi /Go, Gq/G11	↓ Adenylyl cyclase ↓ cAMP PLC-β activation Intrinsic cation channel (Ca2+ and Na+)	Adenosine, Cl-IB-MECA, IB-MECA, MRS5698, NECA, R-PIA, CGS21680	MRS1191, MRS1523,MRS1171, MRS1220, DPCPX,VUF8504, ZM211385, cafeine, theophyline

Adenosine receptors as drug targets

Increasing evidence demonstrate that adenosine receptors may be putative therapeutic targets for countless disease conditions (Borea et al. 2018; Borea et al. 2016; Lopes et al. 2021; Bilkei-Gorzo et al. 2008). Several compounds are currently undergoing clinical trials for the treatment of cardiovascular diseases (A_1 and A_{2A}), pain (A_1), wound healing (A_{2A}), diabetic foot ulcers (A_{2A}), colorectal cancer (A_3) and rheumatoid arthritis (A_3).

A_{2A}R agonists have therapeutic potential because A_{2A} receptor activation produces vasodilatation and reduces inflammatory processes. Several A_{2A}AR antagonists have been studied for the treatment of Parkinson's disease (PD), Alzheimer's disease (AD) and depression. The A_{2A}AR subtype mediates strong immunosuppressive effects and therefore A_{2A}AR antagonists are currently being developed for the immunotherapy of cancer (De Filippo et al. 2020; Yan et al. 2003)

Notwithstanding this, there are still a limited number of adenosinergic drugs on the market. Adenosine is currently used clinically (in the form of generic drugs Adenocard® and Adenoscan®) for the treatment of supraventricular tachycardia, to produce controlled hypotension and for cardiac perfusion imaging, while adenosine-related drugs, like dipyridamole and methotrexate, exert their main effects by altering the extracellular concentrations and signaling of adenosine (Peleli et al. 2017). On the other hand, the European Union approved the AR antagonist caffeine citrate (Gencebok®) for the treatment of sleep apnea of prematurity and the A_{2A}AR agonist, regadenoson (Rapiscan®) for myocardial perfusion imaging (Borea et al., 2018). The European Medicines Agency recognized the selective A_3AR agonist, 2-chloro-N^6-(3-iodobenzyl) adenosine-5'-N-methyluronamide (Cl-IB-MECA; Namodenosone®) as an orphan drug for the treatment of the hepatocellular carcinoma (EMA, 2015). The A_{2A}AR receptor antagonist, istradefylline (Nourianz®) was approved in 2013 for the treatment of Parkinson's disease in Japan and very recently by the US Food and Drug Administration (FDA) for the same purpose. Efforts are being made to increase the number of clinical trials targeting adenosine signaling. There are currently 284 open clinical trials designed

to evaluate the role of adenosine-related drugs for the treatment of various high-impact diseases, including neurodegenerative diseases, autoimmune, cancer, cardiovascular diseases, chronic pain, and COVID-19 (see clinicaltrial.org)(Yan et al. 2003).

The design of new pharmacological efficient and safe approaches to target the adenosine system may have considerable therapeutic potential but are also associated with many challenges (Peleli et al. 2017; Yan et al. 2003). Adenosine receptors have a widespread distribution and are present in virtually all cells, so that it is highly possible that a certain adenosine receptor subtype is present not only in the target cell(s) involved in a disease condition, but also in cells that are involved in many diverse physiological processes (Chen et al. 2013). On way to circumvent this constrain and to provide some degree of tissue specificity while decreasing unwanted systemic side effects is by modifying the local concentrations of endogenous adenosine, so that only nearby adenosine receptors become activated. This can be achieved in several ways. The use of inhibitors of adenosine deaminase (e.g. deoxycoformycin) and/or of the adenosine uptake system (with dipyridamole and ticagrelor) has provide some benefit in epilepsy, diabetes and cardiovascular diseases (Chen et al. 2013). Alternatively, one can use AMP-derived prodrugs that only became activated when the target adenosine receptor is close to the ecto-5'-nucleotidase / CD73 enzyme (Jacobson et al. 2016).

1.4 PURINERIC SIGNALING IN FASCIA: THE ROLE OF ADENOSINE

Fascia is considered our largest sensory organ; it is densely innervated and plays relevant roles in scarring, tissue remodelling, inflammation and pain perception (Correa-Gallegos et al. 2019; Cronstein 2011a; Sawynok 2016). Fibroblasts remodeling is essential for normal fascial function, while their plasticity is crucial for adaptations to injury. Notwithstanding this, chronic deformation and/or stiffness of the myofascial system, for instance due to excessive collagen deposition, favor mechanoreceptors to become

nociceptors thus fostering the emergence of chronic pain. On the other hand, decreased stress due to immobilization or hypomobility can also be harmful to fascia by favoring connective tissue atrophy. It has long been known that scar tissue is a common cause of chronic pain and emerging evidence suggest that connective tissue becomes thicker and less compliant in chronic pain patients, possibly as a result of chronic inflammation and fibrosis (Goldman et al. 2013; Langevin et al. 2009; Langevin, Fox, et al. 2011a; Langevin et al. 2004).

Increases in adenosine production in response to stressful stimuli might have a dual modulatory role in tissue homeostasis. While adenosine first acts as an alarm molecule, reporting injury to surrounding cells in order to trigger protective responses, persistent accumulation of adenosine may lead to maladaptive organ responses, as observed in chronic inflammation, fibrosis, and/or delayed tissue healing conditions (Chan et al. 2006; Chen et al. 2013; Cronstein 2011a). Moreover, the purinergic system through the constitutive release of ATP, extracellular breakdown to adenosine and, subsequent, activation of P1 receptors has been related to the benefits of non-pharmacological treatments, such as acupressure, acupuncture and moxibustion, to deal with myofascial pain conditions (Burnstock 2009; Langevin 2014b). Burnstock hypothesized that skin deformation by the insertion and twisting of needles used in acupuncture causes the local release of huge ATP amounts by keratinocytes and subcutaneous fibroblasts. Extracellular ATP turns on P2X3 and P2X2/3 receptors located in sensory nerve endings of the skin, which activation convey information to pain centers in the central nervous system to initiate feedback control mechanism (Burnstock 2009). Alternatively, ATP released at acupoints may be enzymatically hydrolyzed to adenosine, which might stimulate inhibitory A_1Rs on primary afferent fibers, thus limiting their firing rate (He et al. 2020).

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 (Pinheiro et al. 2013b; Goldman et al. 2013; Langevinet al. 2013; Pinheiro et al. 2013a), very little attention has been given to the effects of their

metabolite, adenosine, operating via P1 receptors (Burnstock 2017b). This is intriguing because the nucleoside is a potent endogenous modulator of neuronal activation, inflammation, and connective tissue repair. Under normal physiological conditions, the extracellular adenosine levels are between 20 and 300 nM. The concentration of the nucleoside raises to the low micromolar range under extreme physiological situations, like intensive exercise or during hypoxic conditions (e.g. high altitude). It can reach high micromolar levels (30 μM) under pathological conditions, such as ischemia (Cronstein et al. 2017).

Mounting evidence suggest that adenosine may be an important modulator of both physiological and pathological processes in the human fascia. Yet there are gaps in our knowledge regarding (1) the expression and function of adenosine receptor subtypes in chronic myofascial pain conditions, particularly in humans, and (2) the influence of confounders, namely consumption of caffeine (a widely used adenosine receptors antagonist), as well as clear control about the disease state and progression at the time of diagnosis.

Clinical relevance of the study

The increasing interest in the relationship between fascial elements and the purinergic system in the context of tissue remodeling, inflammation and fibrosis is promising given to the fact that novel putative pharmacological tools to treat myofascial painful syndromes might emerge. Therefore, there is an urgent need to understand the molecular mechanisms underlying the purinergic cell communication signals between fibroblasts and other cellular players within fascia in order to uncover novel targets.

Taking into consideration that fascia is a mechano-sensitive organ bridging all parts of the body which is composed mainly by fibroblasts interacting with large number of neurons operating proprioception and nociception (Bordoni et al. 2015; Kumka et al. 2012), one can easily understand that disorganization of this complex syncytial network structure may affect health condition in many different ways (Adstrum et al. 2017b). Therefore, we foresee that investigating the molecular mechanisms underlying fascial fibroblasts

central signalling role might contribute to unravel alterations of the whole-body system in myofascial syndromes, like fibromyalgia. To understand the purinergic signaling mechanisms that intermediate connective tissue remodeling under stressful and non-stressful conditions, now focusing on the role of adenosine, we decided to isolate fibroblasts as these cells are the main cellular constituent of fascia.

Aim

This study was designed to fill the gaps in our knowledge regarding the following issues: [1] the controversy concerning the participation of adenosine in human fibroblasts involved in wound healing, dermal fibrosis, and myofascial pain, focusing on the pathways responsible for its production / inactivation and function via P1 receptor subtypes; [2] the role of adenosine originated from the breakdown of ATP released when human fibroblasts are challenged by inflammatory mediators (e.g. histamine, bradykinin); [3] the modulatory role of adenosine in the expression and function of ATP-releasing connexin-43 (Cx43)- and pannexin-1 (Panx1)-containing hemichannels as another way to control human fibroblast cells function amenable to pharmacological intervention.

CHAPTER 2. ORIGINAL RESEARCH PAPERS

PAPER 1:

Opposing Effects of Adenosine and Inosine in Human Subcutaneous Fibroblasts May Be Regulated by Third Party ADA Cell Providers

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Opposing Effects of Adenosine and Inosine in Human Subcutaneous Fibroblasts May Be Regulated by Third Party ADA Cell Providers

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Abstract: Human subcutaneous fibroblasts (HSCF) challenged with inflammatory mediators release huge amounts of ATP, which rapidly generates adenosine. Given the nucleoside's putative relevance in wound healing, dermal fibrosis, and myofascial pain, we investigated the role of its precursor, AMP, and of its metabolite, inosine, in HSCF cells growth and collagen production. AMP (30 μM) was rapidly ($t\frac{1}{2}$ 3 ± 1 min) dephosphorylated into adenosine by CD73/ecto-5′-nucleotidase. Adenosine accumulation ($t\frac{1}{2}$ 158 ± 17 min) in the extracellular fluid reflected very low cellular adenosine deaminase (ADA) activity. HSCF stained positively against A_{2A} and A_3 receptors but were A_1 and A_{2B} negative. AMP and the A_{2A} receptor agonist, CGS21680C, increased collagen production without affecting cells growth. The A_{2A} receptor antagonist, SCH442416, prevented the effects of AMP and CGS21680C. Inosine and the A_3 receptor agonist, 2Cl-IB-MECA, decreased HSCF growth and collagen production in a MRS1191-sensitive manner, implicating the A_3 receptor in the anti-proliferative action of inosine. Incubation with ADA reproduced the inosine effect. In conclusion, adenosine originated from extracellular ATP hydrolysis favors normal collagen production by HSCF via A_{2A} receptors. Inhibition of unpredicted inosine formation by third party ADA cell providers (e.g., inflammatory cells) may be a novel therapeutic target to prevent inappropriate dermal remodeling via A_3 receptors activation.

Keywords: human subcutaneous fibroblasts; inosine; adenosine A_{2A} receptor; adenosine A_3 receptor; exchange protein activated by cyclic AMP (EPAC) pathway; cells proliferation; collagen production

1. Introduction

Subcutaneous connective tissue disorganization is a common feature in patients with chronic pain, possibly as a result of tissue inflammation and fibrosis [1–3]. Chronic deformation of the myofascial system such as increased deposition of type I collagen causes mechanoreceptors to become nociceptors and may develop chronic pain. Fibroblasts represent the base of the fascial system, a connective tissue structure that covers and affects every area of the body [4]. These cells play a key role in transmitting nociceptive information, which is useful for proper functioning of the body system.

Data from previous studies indicate that purines may potentially play a role in subcutaneous inflammation, nociception, and connective tissue remodeling, namely due to the activation of nucleotide-sensitive P2 purinoceptor subtypes [5–8]. However, little information is available regarding the expression and the function of P1 purinoceptor subtypes in the human subcutaneous connective tissue (see, e.g., [9]; reviewed in [10]). These receptors are specifically activated by adenosine, which is the main metabolite of the ectonucleotidase cascade. Our group demonstrated that extracellular ATP and ADP are broken down by membrane-bound NTPDase1 (also called CD39 or apyrase, EC 3.6.1.5) and NTPDase2 (CD39L1, EC 3.6.1.3) into AMP at the surface of human subcutaneous fibroblasts (HSCF), which may be then promptly dephosphorylated into adenosine by ecto-5′-nucleotidase (also called CD73, EC 3.1.3.5) [7,8]. Alternatively, adenosine can be released as such from most cell types via equilibrative nucleoside transporters [11]. The extracellular adenosine accumulation is limited (30–300 nM range) by cellular reuptake and/or deamination into inosine by adenosine deaminase (ADA, EC 3.5.44) [11].

Mounting evidence indicates that extracellular adenosine levels increase dramatically in tissues submitted to stressful conditions, such as ischemia, hypoxia, and inflammation. The nucleoside is a potent endogenous modulator of cardiovascular function, neuronal activation, inflammation, and connective tissue repair. However, excessive extracellular adenosine accumulation in the dermis (and in many other locations) may lead to scar progression and fibrosis [12]. Thus, increasing adenosine production in response to stressful stimuli might have a dual modulatory role in tissue homeostasis [13]. While adenosine first acts as an alarm molecule reporting injury to surrounding cells in order to trigger protective responses, persistent accumulation of adenosine may lead to maladaptive organ responses, as observed in chronic inflammation, fibrosis, and/or delayed tissue healing.

Adenosine effects are mediated by differential activation of four G protein-coupled P1 purinoceptors, namely A_1, A_{2A}, A_{2B}, and A_3; each one may undertake a specific role [14,15]. Depending on the tissue, evidence shows that activation of the same receptor subtype may cause opposing effects. For instance, the adenosine A_{2B} receptor inhibits fibrosis in the heart while promoting fibrosis of the lung [16]. In the skin, the liver, and the lungs, both A_{2A} and A_{2B} receptors favor fibrosis (reviewed in [12]). Nevertheless, the dynamics of adenosine receptor expression and activation in inflammatory conditions and tissue remodeling/fibrosis are still controversial [17]. The putative therapeutic impact of A_1 and A_{2A} receptor agonists in myofascial pain is challenged by significant cardiovascular side effects [18]. On the other hand, identification of novel, powerful, and selective A_3 receptor ligands opens new avenues for elucidation of their therapeutic potential in chronic myofascial conditions [14], but this is weakened by scarcity of studies about their expression and function in fibroblasts of the human subcutaneous tissue [19,20].

Taking into consideration (1) that controversy still exists regarding the participation of adenosine in wound healing, dermal fibrosis, and myofascial pain, and (2) that the nucleoside is promptly generated from the extracellular catabolism of released adenine nucleotides by human fibroblasts challenged with inflammatory mediators (e.g., histamine, bradykinin), we decided to investigate the long-term role of the adenosine precursor, AMP, and its deamination metabolite, inosine, in the proliferation and the collagen production by fibroblasts of the human subcutaneous tissue.

2. Experimental Procedures

2.1. Cell Cultures

Human fibroblasts were isolated from the subcutaneous tissue of male organ donors (45 ± 6 years old, n = 36) with no clinical history of connective tissue disorders. All subjects gave their informed consent for inclusion before they participated in the study. The protocol was approved by the Ethics Committee of Hospital Geral de Santo António SA (University Hospital) and of Instituto de Ciências Biomédicas de Abel Salazar (Medical School) of University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Subcutaneous tissues were maintained at 4–6 °C 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 h after being harvested [21]. 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 37 °C 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.

2.2. Human CD73 Antibody Production

Antibodies to human CD73 were generated by cDNA immunization in Hartley guinea pigs and were validated (http://ectonucleotidases-ab.com/documents/human5-nucleotidase_GP.pdf).

2.3. Immunocytochemistry

Human fibroblasts were seeded in chamber slides at a density of 2.5×10^3 cells/mL and allowed to grow for 7 and 28 days. Cultured cells were fixed in 4% paraformaldehyde (PFA) in PBS for 10 min, washed 3 times in PBS (10 min each), and subsequently incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), and 0.1% Triton X, 0.05% NaN_3) for 1 h. Primary antibodies diluted in blocking buffer II (5% FBS, 1% BSA, 0.1% Triton X, 0.05% NaN_3) were applied [mouse anti-porcine vimentin (1:75, DAKO); rabbit anti-human collagen I (1:50, AbDSerotec, Kidlington, UK); rabbit anti-rat A_1 1:50, and rabbit anti-human A_{2B} (1:75, Chemicon, Temecula, CA, USA); rabbit anti-canine A_{2A} (1:100, Alpha Diagnostic, San Antonio, TX, USA); rabbit anti-human A_3 (1:150, Alomone, Jerusalem, Israel); and guinea-pig anti-human CD73 h5′NT-2_C(I4) (1:300, available at http://ectonucleotidases-ab.com)], and the slides were incubated overnight at 4 °C. After incubation, cells were washed 3 times in PBS 1× (10 min each). The donkey anti-rabbit IgG Alexa Fluor 488, the donkey anti-mouse IgG Alexa Fluor 568, the donkey anti-guinea pig IgG Alexa Fluor 568, and the donkey anti-goat IgG Alexa Fluor 633 secondary antibodies (Molecular Probes, Eugene, OR, 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 4 °C. 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 (Supplementary Figure S1). Observations were performed and analyzed with an Olympus FV1000 confocal microscope (Olympus FV1000, Tokyo, Japan) [7,8,22].

2.4. SDS-PAGE and Western Blotting

Human subcutaneous fibroblasts (HSCF) were seeded in chamber slides at a density of 6.0×10^3 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% bromphenol blue, 20% glycerol, and 10% 2-mercaptoethanol, pH 6.8 at 70 °C for 10 min), subjected to electrophoresis in 10% SDS-polyacrylamide gels, and electrotransferred onto PVDF membranes (MilliPore, Burlington, MA, USA). Protein loads were 150 µg for $A_{2A}R$, A_3R, and CD73. 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 A_3R (1:200, Alomone, Jerusalem, Israel), rabbit anti-human $A_{2A}R$ (1:200, Alpha Diagnostics, San Antonio, TX, USA), and rabbit anti-CD73 (1:500; h5'NT-2_L I5) available at http://ectonucleotidases-ab.com) in the above blocking buffer overnight at 4 °C. 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 (Abcam Plc, Cambridge, UK) secondary antibody for 60 min at room temperature. For normalization purpose, membranes were incubated with mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:200; 37 kDa Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-β-actin (1:5000; 42 kDa; Abcam Plc, Cambridge, UK), and rabbit anti-β-tubulin (1:2500; 50 kDa; Abcam Plc, Cambridge, UK) antibodies following the procedures described above. The antigen-antibody complexes were visualized 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).

2.5. Enzymatic Kinetic Experiments and HPLC Analysis

After a 30 min equilibration period at 37 °C, HSCF grown for 11 days were incubated with 3 or 30 µM of AMP or adenosine, which was added to the culture medium at zero time. Samples (75 µL) were collected from each well at different times up to 30 min for high-performance liquid chromatography (HPLC, LaChrome Elite, Merck, Germany) analysis of the variation of substrate disappearance and product formation [7,8,22]. Adenosine catabolism analysis was performed at room temperature with a LiChrospher 100 RP-18 (5 µm) column (Merck) by isocratic reverse-phase HPLC-UV set at 254 nm. The eluent was composed of 91% of 100 mM KH2PO4, pH = 7, and 9% of methanol during 10 min with a constant rate flow of 1.25 mL/min. Under these conditions, the retention times of adenosine and its metabolites were as follows: adenosine (8.03 min), inosine (2.73 min) and hypoxantine (1.78 min). For the AMP catabolism analysis, we used a linear gradient (100% 100 mM KH_2PO_4, pH 7, to 100% 100 mM KH_2PO_4, pH 7, 30% methanol) for 10 min with a constant rate flow of 1.25 mL/min. Under these conditions, the retention times of metabolites were as follows: AMP (2.17 min), hypoxanthine (3.07 min), inosine (5.09 min), and adenosine (7.51 min).

Concentrations of the substrate and the products were plotted as a function of time (progress curves). The following parameters were analyzed for each progress curve: half-life time ($t\frac{1}{2}$) of the initial substrate, time of appearance of the different concentrations of the products, concentration of the substrate, or any product remaining at the end of the experiment. The spontaneous degradation of AMP and adenosine at 37 °C in the absence of the cells was negligible (0–3%) over 30 min. At the end of the experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (LDH, EC 1.1.1.27) activity. The negligible activity of LDH in the samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental period.

2.6. Cell Viability/Proliferation

Viability/proliferation studies included the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [7,8]. HSCF were seeded in flat bottom 96 well plates at a density of 3×10^4 cells/mL and cultured in supplemented DMEM. Cell cultures were routinely monitored by phase contrast microscopy and characterized at days 1, 7, 14, 21, and 28. The MTT assay consists of the reduction of MTT to a purple formazan reaction product by viable cells. In the last 4 h of

each test period, cells were incubated with 0.5 mg/mL of MTT for 4 h in the conditions referred above. The medium was carefully removed, decanted, and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer (Synergy HT, BioTek, Vermont, VT, USA). Results were expressed as A/well.

2.7. Total Collagen Determination

Collagen determination was performed using the Sirius Red colorimetric assay that stains equally well collagen types I and III [23], which are the two main collagen types existing in the human skin, normally at a ratio of 4:1 [24]. HSCF were cultured as described for the viability/proliferation studies. 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. Results were expressed as A/well.

2.8. Materials and Reagents

Amphotericin B, bovine serum albumin (BSA), Dulbecco's Modified Eagle's Medium (DMEM), ethylene diaminetetraacetic acid (EDTA), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffered saline system (PBS), and type I collagenase, as well as 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene-propanoic acid hydrochloride (CGS 21680), inosine (hypoxanthine 9-β-D-ribofuranoside), adenosine 5′-monophosphate (AMP) sodium salt, adenosine deaminase (ADA type X from bovine spleen, buffered aqueous glycerol solution), Concanavalin A (type IV from *Canavalia ensiformis*, Jack bean), dipyridamole, 9-(tetrahydro-2-furanyl) -9H-purin-6-amine,9-THF-Ade (SQ 22,536), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfon amide dihydrochloride (H-89) dihydrochloride hydrate, α-[2-(3-chlorophenyl) hydrazinylidene]-5-(1,1 -dimethylethyl)-b-oxo-3 isoxazolepropanenitrile (ESI-09), and 3-ethyl-5-benzyl-2-methyl-4-phenylethy nyl-6-phenyl-1,4-(±)-dihy dropyridine-3,5-dicarboxylate (MRS 1191) were purchased from Sigma-Aldrich. The 2-(2-Furanyl)-7-[3-(4-methoxyphenyl-)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]p -yrimidin-5-amine (SCH 442416) and 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (2Cl-IB-MECA) were obtained from Tocris Cookson Inc, Dimethylsulphoxide (DMSO) and glacial acetic acid were obtained from Merck and Bouin's solution was acquired from Panreac. CGS 21680, 2Cl-IB-MECA, Dipyridamole, MRS119, ESI-09, H-89, and SCH 442416 were prepared in dimethyl sulfoxide (DMSO). All other drugs were prepared in distilled water or culture medium. All stock solutions were stored as frozen aliquots at −20 °C. Dilutions of these stock solutions were made daily, and appropriate solvent controls were done. No statistically significant differences between control experiments made in the absence or in the presence of DMSO at the maximal concentration used (0.05% *v/v*) were observed. The pH of the solutions did not change by the addition of the drugs in the maximum concentrations applied to the preparations. The 96-well tissue culture plates were purchased from Corning; chamber slides were from Nunc.

2.9. 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 8.3.0 software (La Jolla, CA, USA). One- or two-way ANOVA followed by the Tukey's multicomparison test was used only if F was significant and there was no variance inhomogeneity; few outliers were identified and removed using the ROUT method with a Q = 1%. $p < 0.05$ (two-tailed) values were considered statistically significant.

3. Results

3.1. Human Subcutaneous Fibroblasts Express Ecto-5′-Nucleotidase/CD73 and Adenosine A_{2A} and A_3 Receptor Subtypes

Human subcutaneous fibroblasts (HSCF) are elongated cells with a characteristic spindle-shaped morphology exhibiting positive immunoreactivity against fibroblast-cell markers, such as vimentin and type I collagen (Figure 1A; see also [7,8]). From the four adenosine receptor subtypes, cultured HSCF showed strong immunoreactivity against A_{2A} and A_3 receptor subtypes, with faint A_{2B} receptor staining and no evidence of the A_1 receptor being present in these cells (Figure 1B). The low immunoreactivity against A_1 and A_{2B} receptors could not be attributed to deficient quality of the antibodies, because positive identification of the two receptors was previously demonstrated by our group in human primary bone marrow stromal cells undergoing osteogenic differentiation using the same experimental procedure and antibodies (anti-A1 #AB1587P and anti-A2B #AB1589P from Chemicon, Temecula, CA, USA) (Supplementary Figure S1; see also [22]).

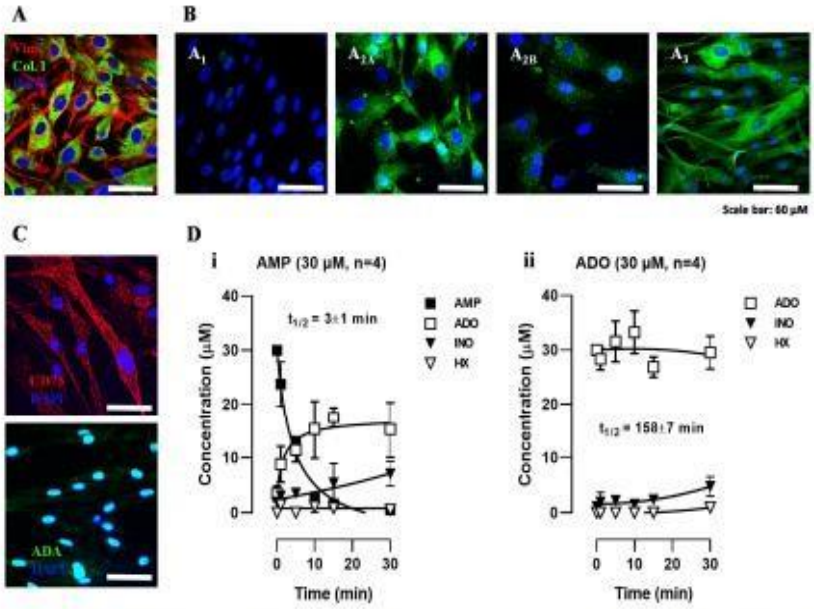


Figure 1. Human subcutaneous fibroblasts (HSCF) express ecto-5′-nucleotidase/CD73, the enzyme responsible for AMP dephosphorylation into adenosine, but lack adenosine deaminase (ADA), resulting in extracellular adenosine accumulation that may signal via co-expressed A_{2A} and A_3 receptor subtypes. Panel **A** shows the immunoreactivity against fibroblast cell markers, vimentin (red), and type I collagen (green). The panel **B** shows that HSCF stain positively against A_{2A} and A_3 receptors (green), with very little amounts of A_{2B} and A_1 receptor subtypes. The panel **C** shows that HSCF are ecto-5′-nucleotidase/CD73 positive ADA negative cells. Nuclei are stained in blue with DAPI; scale bar is 60 µm. Micrographs were obtained from at least three different individuals with a laser scanning confocal microscope using the same acquisition settings. Panel **D** shows the time course of the extracellular catabolism of AMP (30 µM, (**i**) and adenosine (ADO, 30 µM, (**ii**) in HSCF cultures allowed to grow for 11 days. AMP and ADO were added to the culture medium at time zero. Samples (75 µL) were collected from each well at indicated times in the abscissa. Each collected sample was analyzed by HPLC to separate and quantify AMP (filled squares), adenosine (open squares), inosine (filled triangles), and hypoxanthine (open triangles). Each point represents pooled data from four individuals; two replicas were performed in each individual experiment. Vertical bars represent SEM and are shown when they exceed the symbols in size. The calculated half-life time ($t\frac{1}{2}$, min) for each initial substrate is shown for comparison.

Figure 1C shows that cultured HSCF expressed significant amounts of ecto-5′-nucleotidase/CD73, the enzyme that is responsible for extracellular AMP dephosphorylation enabling adenosine formation from released adenine nucleotides. The lack of adenosine deaminase (ADA) staining at the plasma membrane of these cells (Figure 1C) suggests that adenosine may accumulate in the extracellular milieu strengthening activation of plasma membrane-bound adenosine receptors.

3.2. Adenosine Formation from AMP Overcomes its Deamination into Inosine Favoring Accumulation of the Nucleoside in HSCF Cultures

Figure 1D illustrates the time course of the extracellular catabolism of AMP and adenosine in cultured HSCF. AMP (30 μM) was rapidly ($t\frac{1}{2}$ 3 ± 1 min, n = 4) dephosphorylated into adenosine by ecto-5′-nucleotidase/CD73; little amounts of inosine were detected 15 min after application of the nucleotide. The progress curve of adenosine (30 μM) disappearance shows that the nucleoside was slowly ($t\frac{1}{2}$ 158 ± 17 min, n = 4) deaminated into inosine with almost no formation of hypoxanthine in HSCF cultures. Inosine reached a maximal concentration of 4 ± 3 μM 30 min after adenosine (30 μM) application. The absence of AMP formation from adenosine suggests that no extracellular adenosine kinase (ADK, E.C. 2.7.1.20) activity was present in HSCF. The stoichiometry of extracellular adenosine disappearance and metabolites formation was kept fairly constant throughout the reaction time period, i.e., the sum of the initial substrate, the adenosine, plus its metabolites was roughly 30 μM in all considered time points, leading to the conclusion that cellular adenosine uptake was irrelevant under the present experimental conditions.

Using a smaller (3 μM) concentration, adenosine was metabolized with a half-life time ($t\frac{1}{2}$) of 40 ± 8 min (n = 4), leading to a maximal inosine concentration of 1 ± 1 μM 30 min after adenosine application (data not shown). The semi-logarithmic representation of progress curves obtained by polynomial fitting of the nucleoside catabolism show a linear pattern ($y = -0.0051x + 0.4578$ and $R^2 = 0.99$ for 3 μM: $y = -0.0015x + 1.4640$ and $R^2 = 0.99$ for 30 μM). Given that the slope of the progress curves decreased upon increasing the concentration of the substrate from 3 to 30 μM, data indicate that the kinetics of the catabolism of adenosine was slower upon increasing the concentration of the nucleoside.

Enzymatic kinetic studies confirm our prediction that HSCF express small amounts of ADA, which is rapidly overcome by adenosine formation from released adenine nucleotides, thus favoring adenosine accumulation with little inosine production in the extracellular milieu.

3.3. AMP Favors Collagen Production via Adenosine A_{2A} Receptors Activation Coupled to the Adenylyl Cyclase (AC)/Exchange Protein Activated by Cyclic AMP (EPAC) Pathway in HSCF

Incubation of HSCF cultures with AMP (30–100 μM) for 28 days concentration-dependently increased ($p < 0.05$) collagen production (Sirius Red assay; Figure 2A) without affecting cells growth (MTT assay; Figure 2B). The pro-fibrotic effect of AMP (100 μM) was fully prevented by the non-nucleotide inhibitor of ecto-5′-nucleotidase/CD73, concanavalin A (0.01 mg/mL) (Figure 2C). Interestingly, the facilitatory effect of AMP on collagen production was more evident at culture days 21 and 28. These time points coincided with increases in the ecto-5′-nucleotidase/CD73 protein content shown by immunocytochemistry and Western blot analysis comparing culture days seven and 28 (Figure 2D).

Results suggest that promotion of collagen production induced by AMP requires progressive extracellular adenosine formation by ecto-5′-nucleotidase/CD73 in mature HSCF. The involvement of co-expressed adenosine receptors on AMP-induced collagen production by HSCF was investigated using subtype-selective A_{2A} and A_3 receptor antagonists, SCH442416 (10 nM) and MRS1191 (10 nM), respectively. The facilitatory effect of AMP (100 μM) on collagen production was prevented upon blocking the A_{2A} receptor with SCH442416 (10 nM) (Figure 2E), while selective blockage of the A_3 receptor with MRS1191 (10 nM) was virtually ineffective (Figure 2F). Data suggest that AMP favors collagen production by HSCF via adenosine A_{2A} receptors activation.

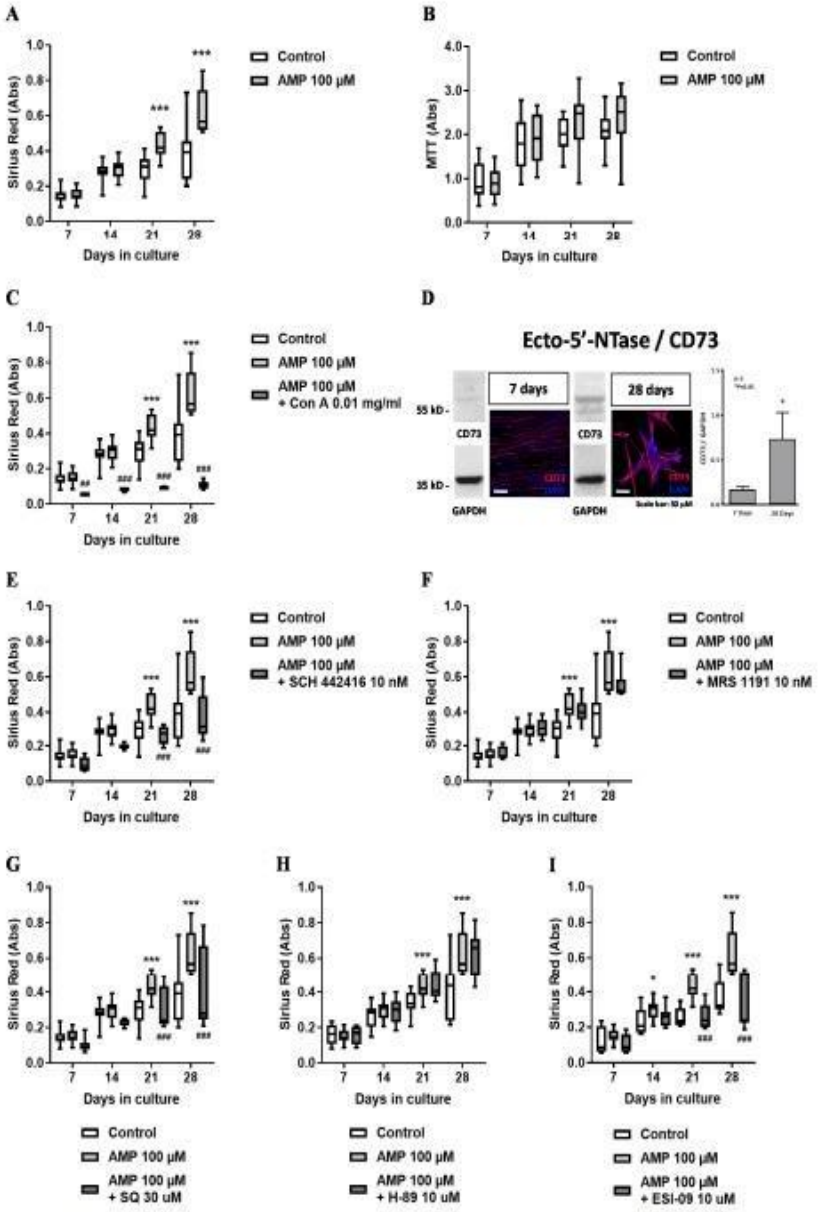


Figure 2. Preferential effect of the adenosine precursor, AMP (100 µM), on collagen production (Sirius Red staining, panel **A**) by human subcutaneous fibroblasts (HSCF) kept in culture for 28 days with no significant effect on cells proliferation/viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, panel **B**). The effect of AMP (100 µM) was prevented by the ecto-5′-nucleotidase/CD73 inhibitor, concanavalin A (0.01 mg/mL, panel **C**), indicating that the nucleotide must be converted into adenosine to exert its pro-fibrotic effect. Panel **D** shows that ecto-5′-nucleotidase/CD73 immunoreactivity increases with the time of the cells in culture, as evidenced both by immunofluorescence confocal microscopy and by Western blot analysis normalized by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content of the cells. Nuclei are stained in blue with DAPI. Shown are representative data from three different individuals; experiments were performed in triplicate. The involvement of A_{2A} receptors coupled to the adenylate cyclase (AC)/exchange protein activated by cyclic AMP (EPAC) pathway was proven by prevention of the pro-fibrotic effect of AMP

with SCH442416 (10 nM, panel **E**), SQ22536 (30 μM, panel **G**), and ESI-09 (10 μM, panel **I**), which blocked the A_{2A} receptor, AC and EPAC, respectively. Blockage of the A_3 receptor and of protein kinase A with MRS1191 (10 nM, panel **F**) and H-89 (10 μM, panel **H**), respectively, was devoid of effect. Boxes and whiskers represent pooled data from at least three different individuals; 4–6 replicas were performed for each individual. * $p < 0.05$ and *** $p < 0.001$ (two-way ANOVA) represent significant differences compared to control conditions in the same individual; ## $p < 0.01$ and ### $p < 0.001$ (two-way ANOVA) represent significant differences compared to the effect of AMP in the same set of experiments.

Next, we set to investigate the intracellular signaling pathway coupling adenosine A_{2A} receptors activation to collagen production by HSCF in the presence of the adenosine precursor, AMP. Most commonly, the A_{2A} receptor couples to the adenylyl cyclase (AC)/cyclic AMP pathway. Selective inhibition of AC with SQ22536 (30 μM; Figure 2G) as well as inhibition of the exchange protein activated by cyclic AMP (EPAC) with ESI-09 (10 μM; Figure 2I), but not of the protein kinase A with H-89 (10 μM; Figure 2H), prevented AMP-induced facilitation of collagen production by HSCF. Blockage of cyclic AMP specific type IV phosphodiesterase with rolipram (300 μM) mimicked the pro-fibrotic effect of AMP (100 μM) on collagen production at culture days 21 and 28 without affecting HSCF cells growth (data not shown). Overall, data suggest that adenosine A_{2A} receptors coupled to the AC/EPAC pathway are involved in the pro-fibrotic effect of AMP in cultured HSCF.

3.4. Effect of Adenosine A_{2A} and A_3 Receptor Agonists on Proliferation/Viability and Collagen Production by HSCF

The facilitatory effect of AMP (100 μM) on collagen production was mimicked by the selective A_{2A} receptor agonist CGS21680C (10 nM) (Figure 3B). Likewise, the A_{2A} receptor agonist also did not modify cell viability/proliferation of HSCF (Figure 3A). The facilitatory effect of CGS 21680 (10 nM) on collagen production by HSCF was prevented by co-application of the selective A_{2A} receptor antagonist, SCH442416 (10 nM) (Figure 3B); neither CGS 21680 (10 nM) alone or in the presence of SCH442416 (10 nM) affected viability/proliferation of HSCF (Figure 3A). Like that observed with AMP (100 μM), the pro-fibrotic effect of CGS 21680 (10 nM) was more evident at culture days 21 and 28, when HSCF exhibited the highest A_{2A} receptor immunoreactivity, as determined by immunocytochemistry and Western blot analysis (Figure 3C).

Given that HSCF co-express the adenosine A_3 receptor throughout the culture period (Figure 4E), but this receptor did not play a role in the pro-fibrotic effect of AMP (Figure 2F), we tested its function on HSCF cell viability/proliferation and collagen production using a highly selective A_3 receptor agonist, 2Cl-IB-MECA (100 nM). Exposure of HSCF to 2Cl-IB-MECA (100 nM) inhibited cell viability/proliferation (Figure 4A) and collagen production (Figure 4B) at culture days 21 and 28. The anti-proliferative effect of 2Cl-IB-MECA had a higher magnitude while becoming evident from culture day 14 onwards upon increasing the concentration of the A_3 receptor agonist from 10 to 100 nM (data not shown). Inhibition of HSCF cells growth by 2Cl-IB-MECA (100 nM) was prevented by blocking the A_3 receptor with MRS1191 (10 nM) (Figure 4A). The action of 2Cl-IB-MECA (100 nM) on collagen production was unaffected by MRS1191 (10 nM) (Figure 4B). Data suggest that, while the adenosine A_3 receptor exerts a predominant anti-proliferative action on HSCF, adenosine formation from released adenine nucleotides activates preferentially A_{2A} receptors to increase collagen production by these cells.

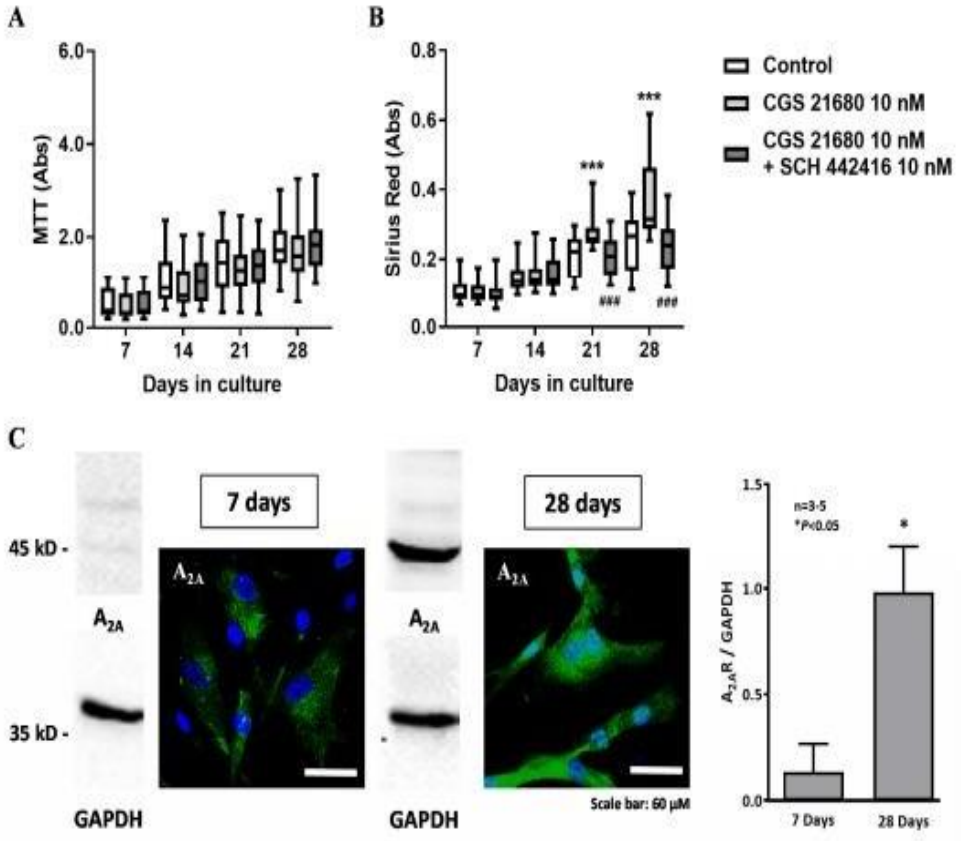


Figure 3. The adenosine A_{2A} receptor agonist, CGS21680 (10 nM), mimicked the effect of AMP on collagen production (Sirius Red staining, panel **B**) while having no effect on human subcutaneous fibroblasts (HSCF) growth (MTT assay, panel **A**). The pro-fibrotic effect of CGS21680 (10 nM) was abrogated by the selective A_{2A} receptor antagonist, SCH442416 (10 nM). Boxes and whiskers represent pooled data from at least three different individuals; 4–6 replicas were performed for each individual. *** $p < 0.001$ (two-way ANOVA) represent significant differences compared to control conditions; ### $p < 0.001$ (two-way ANOVA) represent significant differences compared to the effect of CGS21680 in the same set of experiments. Panel **C** shows that A_{2A} receptor expression (green) increases from day seven to 28, as evidenced both by immunofluorescence confocal microscopy and by Western blot analysis normalized by the GAPDH content of the cells. Nuclei are stained in blue with DAPI. Shown are representative data from three (7 days) to five (28 days) different individuals; experiments were performed in triplicate. * $p < 0.05$ (Student's *t*-test) represent significant differences compared to culture day seven.

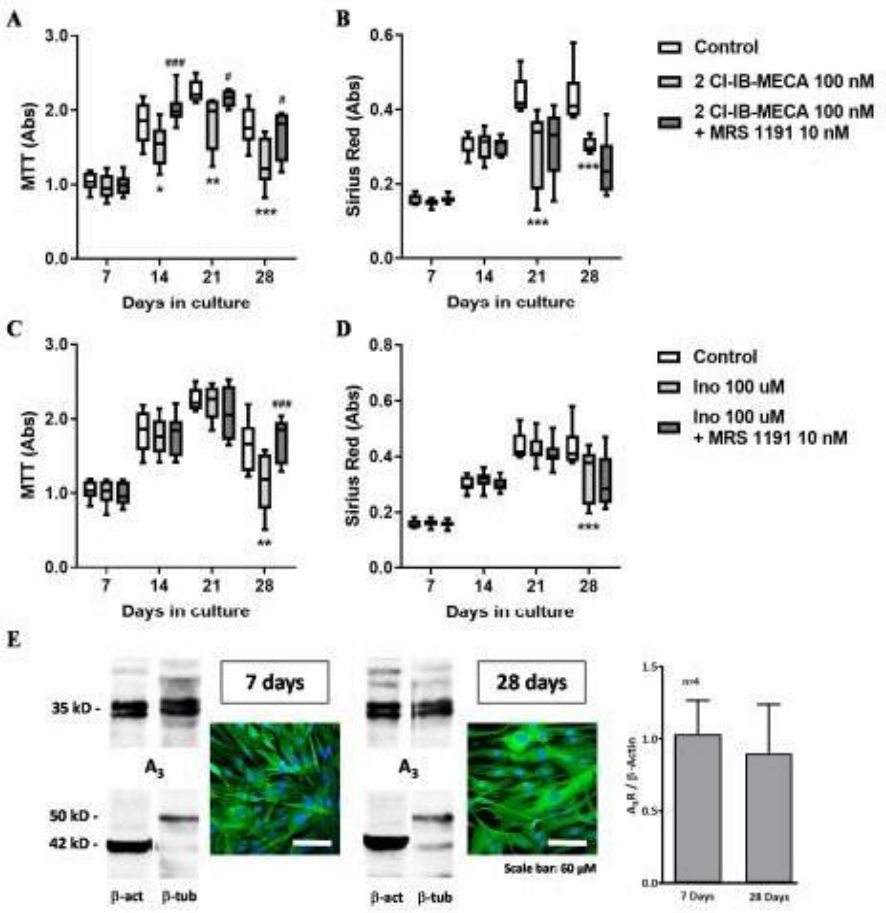


Figure 4. The adenosine metabolite, inosine (100 μM), mimicked the inhibitory effect of the adenosine A_3 receptor agonist, 2Cl-IB-MECA (100 nM), on cells proliferation/viability (MTT assay, **A** and **C**) and collagen production (Sirius Red staining, **B** and **D**) by human subcutaneous fibroblasts (HSCF) grown in culture for 28 days. Inhibition of HSCF cells proliferation by 2Cl-IB-MECA (100 nM, **A**) and inosine (100 μM, **C**) was attenuated by MRS1191 (10 nM), but the selective A_3 receptor antagonist did not affect the inhibitory role of 2Cl-IB-MECA (100 nM, **B**) and inosine (100 μM, **D**) on collagen production. Boxes and whiskers represent pooled data from at least three different individuals; 4–6 replicas were performed for each individual. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (two-way ANOVA) represent significant differences compared to control conditions; $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ (two-way ANOVA) represent significant differences compared to the effect of 2Cl-IB-MECA in the same set of experiments. Panel **E** shows that A_3 receptor expression (green) was kept fairly constant with time of the cells in culture, as evidenced both by immunofluorescence confocal microscopy and by Western blot analysis normalized either by β-actin (left hand-side blots) or β-tubulin (right hand-side blots) content of the cells. Nuclei are stained in blue with DAPI. Shown are representative data of cells from four different individuals cultured for 7 and 28 days, respectively; experiments were performed in triplicate.

3.5. Inosine Exerts an Anti-Proliferative Role on HSCF through Activation of A_3 Receptors, but its Formation Requires Exogenous ADA Application

Although inosine was classically considered to be an inactive metabolite of adenosine, activation of adenosine A_3 receptors by inosine was previously demonstrated [25–28]. The lack of effect of AMP on HSCF cells growth despite the presence of adenosine A_3 receptors together with the low expression

and activity of ADA in these cells that limit inosine formation from adenosine in these cultures led us to test whether inosine could be the endogenous ligand of A_3 receptors in HSCF.

Incubation of HSCF with inosine (100 μM) decreased cells viability/proliferation (Figure 4C) and collagen production (Figure 4D) at culture day 28. The inhibitory action of inosine (100 μM) mimicked the effect of the A_3 receptor agonist, 2Cl-IB-MECA (100 nM) (Figure 4A,B). Likewise, the anti-proliferative effect of inosine (100 μM) was also prevented by MRS1191 (10 nM) (Figure 4C), but the A_3 receptor antagonist did not affect inhibition of collagen production by inosine (100 μM) (Figure 4D). Results suggest that inosine exerts a predominant inhibitory effect on HSCF cell growth by binding to the adenosine A_3 receptor.

If our theory is correct, supplementation of culture media with ADA would facilitate adenosine conversion to inosine in HSCF cultures leading to a reduction in cells growth by preferential activation of A_3 receptors, given that activation of this receptor subtype dependent on sufficient amounts of inosine generated in the cultures. Figure 5 shows that exogenous application of ADA (0.5 U/mL) significantly ($p < 0.05$) decreased HSCF cells viability/proliferation (MTT assay; Figure 5A) without greatly affecting collagen production (Sirius Red assay; Figure 5B) by these cells.

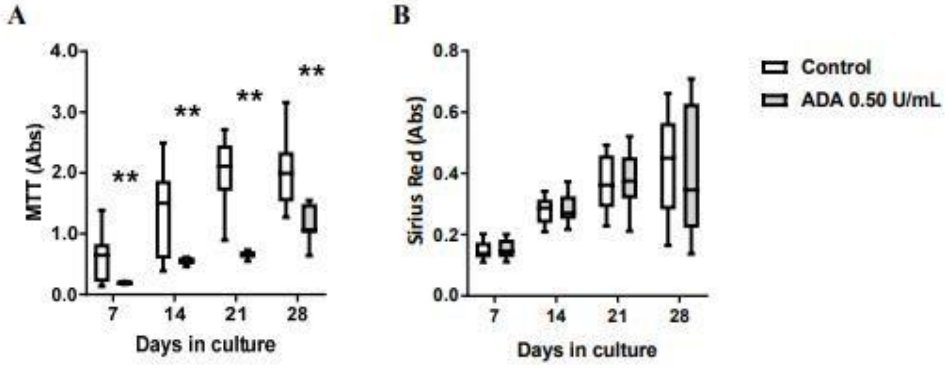


Figure 5. Adenosine deaminase (ADA, 0.5 U/mL) decreases the viability/proliferation (MTT assay, **A**) of human subcutaneous fibroblasts (HSCF) without modifying collagen production (Sirius Red assay, **B**). Boxes and whiskers represent pooled data from at least three different individuals; 4–6 replicas were performed for each individual. ** $p < 0.01$ (one-way ANOVA) represent significant differences compared to control conditions in the same individual.

4. Discussion

Adenosine is present in most biological fluids. Under basal conditions, the extracellular adenosine concentration is maintained within certain limits (30–300 nM), which are normally above its intracellular concentration because the nucleoside is freely phosphorylated by high affinity intracellular adenosine kinase. Under stressful conditions, extracellular adenosine levels dramatically increase to the micromolar range, allowing the nucleoside to exert multiple actions, namely to protect against ischemic and inflammatory insults as well as to regulate neuronal excitability and the release of neuro- and vasoactive substances [29]. Adenosine most often originates from the extracellular catabolism of adenine nucleotides (danger molecules) released from stressed and/or damaged cells (reviewed in [30]). In previous studies, we demonstrated that fibroblasts of the human subcutaneous tissue respond to inflammatory mediators, such as bradykinin and histamine, by releasing substantial amounts of ATP into the extracellular medium through opening of hemichannels containing connexin-43 and/or pannexin-1 subunits [7,8].

Once released, adenine nucleotides are broken down by NTPDase1 and NTPDase2 bound to the plasma membrane of HSCF [7]. NTPDase1 and NTPDase2 hydrolyze extracellular tri- and diphosphonucleotides with ATP/ADP ratios of ~1–2:1 and ~10–40:1, respectively [31]. The kinetic

analysis of the extracellular catabolism of ATP and ADP in HSCF indicates that adenine nucleotides were metabolized with a ratio of ~1.5 (ATP):1(ADP), which is compatible with NTPDase1 being the most effective enzyme in this context [7,8]. The hydrolysis of ATP and ADP by NTPDase1 yielded AMP, which could then be dephosphorylated to adenosine by ecto-5′-nucleotidase/CD73 [32]. The results presented here show that HSCF (as with other mesenchymal-originated cells; see [22,33]) exhibited increasing amounts of the ecto-5′-nucleotidase/CD73 protein with time of the cells in culture. This feature supports a faster conversion of AMP to adenosine than that occurring in many other tissue samples, where ecto-5′-nucleotidase/CD73 is normally the rate limiting enzyme of the ectonucleotidase cascade [34–39]. Interestingly, overexpression of subcutaneous NTPDase1 was reported in a model of chronic inflammation [40]. Inflammatory infiltrates, including T lymphocytes endowed with the ecto-5′-nucleotidase/CD73 enzyme, may additionally contribute to fast AMP dephosphorylation and surplus adenosine formation, leading to unpredictable P1 receptor-mediated responses in the inflamed subcutaneous tissue.

Apart from adenosine formation rate via the ectonucleotidase cascade, the extracellular concentration of the nucleoside near its receptors is strictly regulated by its inactivation pathways, both cellular uptake and ADA. Systemic elevation of adenosine levels in ADA knockout mice has been related to signs of fibrosis in the lungs, the liver, and the kidneys, suggesting a pro-fibrotic trend when ADA is scarce [41]. Moreover, the pharmacological blockade of the A_{2A} receptor prevented dermal fibrosis in this animal model [42]. The enzymatic kinetic experiments performed in this study together with protein detection by immunofluorescence and Western blot analysis show that HSCFs express very little amounts of ADA. Furthermore, the stoichiometry of extracellular adenosine disappearance and formation of its metabolites, inosine and hypoxanthine, was kept fairly constant throughout time, indicating that adenosine cellular uptake by HSCF is negligible. Thus, adenosine formation from released adenine nucleotides may be unbalanced by the lack of nucleoside inactivation mechanisms, ADA and cellular uptake, favoring adenosine accumulation with little inosine (and hypoxanthine) production in the HSCF microenvironment (Figure 6).

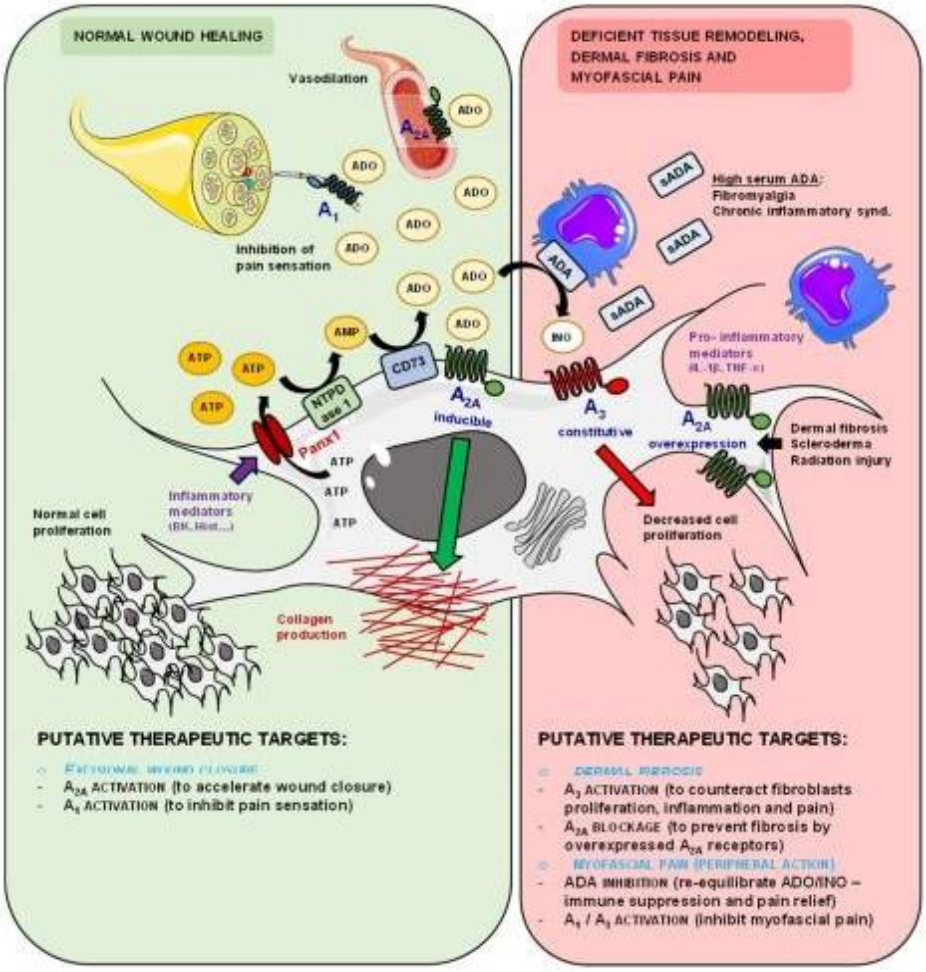


Figure 6. Fine-tuning control of adenosine (ADO) A_{2A} and A_3 receptors activation in human subcutaneous fibroblasts (HSCF) and its putative pathophysiological implications in wound healing, dermal fibrosis, and myofascial pain. Stressed HSCF release huge amounts of ATP to the extracellular medium, actions of which are rapidly terminated by NTPDase1, resulting in the formation of AMP. HSCFs exhibit high amounts of ecto-5′-nucleotidase/CD73, leading to fast dephosphorylation of AMP into ADO, which tends to accumulate in the extracellular milieu due to a very low ADO deaminase (ADA) activity in these cells. The close proximity between ecto-5′-nucleotidase/CD73 and the A_{2A} receptor and their parallel induction during HSCF cells maturation favor activation of the A_{2A} receptor by ADO generated endogenously from ATP extracellular breakdown. Thus, during normal wound closure, ADO produced by differentiated HSCF may contribute to normal collagen production and vasodilation (via A_{2A} receptors activation) while reducing pain sensation through stimulation of A_1 inhibitory receptors on peripheral nerve afferents. A different scenario may, however, occur due to unpredicted inosine (INO) formation as a consequence of ADA-bearing inflammatory cell infiltrates as well as in conditions exhibiting high serum ADA levels, such as fibromyalgia and chronic inflammatory states. INO, via constitutively expressed A_3 receptors, decrease HSCF growth and consequently collagen production. Thus, the novel anti-fibrotic effect of INO together with the anti-inflammatory

and the anti-nociceptive properties of highly selective A_3 receptor agonists undergoing clinical trials may be useful for the treatment of dermal fibrosis and myofascial pain associated with inappropriate subcutaneous tissue remodeling. Considering that overexpression of A_{2A} receptors is a common feature in fibroblast malignancies (e.g., dermal fibrosis, scleroderma, radiation dermal injury), association between A_3 receptor agonists and A_{2A} receptor antagonists may also be proposed. On the other hand, re-equilibration of ADO/INO concentration ratio using ADA blockers may be helpful to promote immune suppression and nociception relief in myofascial pain conditions with or without A_1 and/or A_3 receptor agonists. See text for additional information. Illustration used elements from Servier Medical Art (http://smart.servier.com).

The exact role of adenosine and of its metabolites in subcutaneous tissue homeostasis/remodeling is still controversial, but it certainly deserves attention given our findings showing a tendency for adenosine accumulation in HSCF cultures (this study) and its putative clinical involvement in chronic inflammation and myofascial pain [1–3]. Evidence suggests that adenosine promotes fibrosis in several organs through different mechanisms associated with distinct patterns of adenosine receptors expression and activation [13,41]. Notwithstanding this, it was also demonstrated that the same adenosine receptor subtype may inhibit or promote fibrosis depending on the target tissue [12]. Adenosine receptors were identified in human dermal fibroblasts, yet, to our knowledge, no previous attempts have been made to characterize the receptor subtypes predominantly involved in the effect of endogenously produced adenosine from adenine nucleotides by human subcutaneous (hypodermal) fibroblasts.

Data from immunofluorescence confocal microscopy show that adenosine A_{2A} and A_3 receptors are the most expressed P1 receptor subtypes in primary cultures of HSCF (first subculture). Despite this, the adenosine precursor AMP consistently increased collagen production by HSCF via preferential adenosine A_{2A} receptors activation without significantly affecting cells growth. This was suggested because AMP-induced collagen production was mimicked by treatment of HSCF with the selective A_{2A} receptor agonist, CGS21680 (K_i = 27 nM in human cells; [43]), applied in the nanomolar concentration range. Moreover, the pro-fibrotic effects of AMP and CGS21680 were both abrogated in the presence of SCH442416, which specifically antagonizes the A_{2A} receptor, but not upon blocking the A_3 receptor with MRS1191. Interestingly, increases in collagen production caused by AMP were more evident at culture days 21 and 28, thus coinciding with the highest ecto-5′-nucleotidase/CD73 and A_{2A} receptor protein amounts in HSCF determined by immunocytochemistry and Western blot analysis. These results suggest that the expression of ecto-5′-nucleotidase/CD73 and A_{2A} receptor proteins is induced during HSCF differentiation by a yet unknown molecular mechanism. A tight association between ecto-5′-nucleotidase/CD73 and the A_{2A} receptor was documented by co-immunoprecipitation and proximity ligation assays [44]. Co-localization of these two proteins was also demonstrated by immunofluorescence confocal microscopy in GFAP-positive astrocytes of the human hippocampus [45]. Therefore, ecto-5′-nucleotidase/CD73 is positioned ideally to promote the A_{2A} receptor activation after conversion of AMP into adenosine (Figure 6). Preferential activation of A_{2A} receptors by adenosine generated from the extracellular breakdown of released adenine nucleotides was observed by our group in other locations besides the central nervous system (e.g., neuromuscular junction, myenteric plexus) [35,46].

The A_{2A} receptor seems to be the dominant adenosine receptor in the skin, where it promotes wound healing and excisional wound closure that is normally accompanied by increased collagen (matrix) deposition [12]. This receptor may also play a role in the pathogenesis of fibrotic malignancies of the skin, such as dermal fibrosis. The adenosine A_{2A} receptor is overexpressed in fibroblasts of scleroderma patients presenting excessive collagen deposition in the skin and the visceral organs [47]. Thus, blockade of the adenosine A_{2A} receptor has been proposed to prevent dermal fibrosis and scarring by reducing the collagen content and its misalignment (reviewed in [24,42]) (Figure 6). Our findings showing that adenosine A_{2A} receptors preferentially mediate AMP-induced collagen production by HSCF fully agree with previous assumptions, yet controversy still exists regarding the downstream intracellular pathways responsible for A_{2A} receptor-mediated effects in HSCF. Here,

we suggest that AMP applied for up to 28 days favors collagen production by HSCF (with only one passage) through activation of adenosine A_{2A} receptors coupled to the AC/EPAC pathway with no changes being detected upon blocking PKA with H-89. Fine-tuning regulation of type I vs type III collagen production by adenosine A_{2A} receptors activation coupled to the cyclic AMP/PKA/EPAC pathway was reported in human dermal fibroblasts [24]. These authors showed a dual role of the A_{2A} receptor agonist, CGS21680, on type I vs. III collagen production via preferential activation of PKA and EPAC2 when the adenosine analogue was used for periods up to 4 h in the nanomolar and the micromolar concentration ranges, respectively. In our study, we used the Sirius Red assay, which does not distinguish between type I and III collagen production [23]. Nevertheless, one may speculate that the preferential coupling of adenosine A_{2A} receptor activation to the EPAC pathway in HSCF is more likely to increase the proportion of type III over type I collagen, as detected in the granulation tissue and immature scars, where the local concentrations of adenine nucleotides and adenosine are elevated [24,48]. This hypothesis is corroborated by the fact that high intracellular cyclic AMP levels achieved by inhibiting the cyclic AMP specific type IV phosphodiesterase with rolipram mimicked the pro-fibrotic role of A_{2A} receptors activation with CGS21680 or the adenosine precursor, AMP.

Although the adenosine A_3 receptor subtype was constitutively expressed in fairly constant amounts throughout time, this receptor did not play a role in the pro-fibrotic effect of AMP in HSCF cultures. This prompted us to investigate the activity of the A_3 receptor in cells viability/proliferation and collagen production using a highly selective A_3 receptor agonist, 2Cl-IB-MECA. Data show that activation of the A_3 receptor with 2Cl-IB-MECA decreased cells growth in a MRS1191-sensitive manner, confirming that adenosine A_3 receptors are fully operative in these cells. Interestingly, exogenously added ADA or the adenosine deamination metabolite, inosine, mimicked the anti-proliferative effect of the A_3 receptor agonist. These findings show here, for the first time, that AMP, through its breakdown into adenosine, exerts a predominant pro-fibrotic action on HSCF via preferential A_{2A} receptors stimulation, but it may decrease cells growth upon increasing ADA activity, resulting in substantial inosine formation and A_3 receptors activation under pathological conditions (Figure 6; see discussion below). It is worth noting that inosine is a putative adenosine A_3 receptor agonist in several preparations [25–28].

The adenosine A_3 receptor activation elicits multiple signaling pathways within a cell ending up to the control of many different roles depending on the cell type and agonists concentrations. Upon activation of the A_3 receptor, (1) the Gα subunit may dissociate from its $G_{\beta\gamma}$, decreasing the catalytic activity of adenylyl cyclase and cyclic AMP production, (2) phospholipase C is activated, leading to Ca^{2+} increase or PI3K, Akt phosphorylation, (3) G protein RhoA and phospholipase D are stimulated, (4) mitogen-activated protein kinase (MAPK) family, such as extracellular regulated kinases (ERKs), MAPK-interacting kinase (MNK), and p38, are modulated, and (5) K_{ATP} channels are opened; downstream these pathways, are a series of transcription factors, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the cAMP response element-binding protein (CREB), the hypoxia-inducible factor 1-alpha (HIF-1α), and the c-myc (reviewed in [18]). Mechanical loading of rat subcutaneous fibroblasts increases adenosine levels and upregulates A_3 receptors expression and signaling through the MAPK signaling pathway, resulting in modification of cells proliferation and immune-related factors production [49]. Among the MAPK family, which is subdivided into extracellular regulated kinases, such as ERK1/2, and stress-activated protein kinases (SAPK), such as p38 and jun-N-terminal kinase (JNK), the ERK1/2 seems to represent the preferential pathway for cell division and proliferation responses. Therefore, one may speculate that this pathway may act downstream A_3 receptors activation, yet further studies are required to elucidate whether this mechanism is involved in the anti-proliferative role of inosine in HSCF.

Overall, data enlighten the idea that adenosine, via preferential activation of A_{2A} receptors in fibroblasts, might be a key player in human subcutaneous connective tissue homeostasis and normal wound repair, while excessive scarring and dermal fibrosis may occur when this receptor is overexpressed (Figure 6). For instance, adenosine A_{2A} receptor antagonists have been proposed to

prevent radiation-induced dermal injury [50]. Disturbed adenosine homeostasis in the subcutaneous tissue may happen upon infiltration by ADA-bearing inflammatory cells and/or in situations occurring with increases in serum ADA levels, such as fibromyalgia and other chronic inflammatory conditions [51]. Under such circumstances, the unpredicted breakdown of adenosine into inosine, and subsequent activation of A_3 receptor in HSCF may favor deficient connective tissue remodeling and failure of its viscoelastic properties. Given the fact that subcutaneous connective tissue forms an interconnected net throughout all body [52,53], increases in tissue tension can waveform propagate the release of adenine nucleotides and inosine formation whenever ADA is available, resulting in global changes in the connective tissue network further restraining its compliance. This feature may be the basis for enhanced peripheral inputs and superfluous nociceptive transmission from peripheral soft tissues, which might be involved in the pathogenesis of chronic myofascial pain (Figure 6).

Paradoxically, the adenosine A_3 receptor was recently implicated in the anti-hyperalgesic effect of adenosine in several chronic pain models [54], mainly through blockage of N-type Ca^{2+} currents and of action potential generation in sensory neurons [55]. Likewise, the role of adenosine in acupuncture-mediated antinociception in human subjects (most probably via A_1 receptors activation) has been proposed by demonstrating that acupuncture needle rotation transiently increases interstitial adenine nucleotides, whereas adenosine accumulates for more one hour in subcutaneous microdialysates [56]. Under such conditions, adenosine most probably originates from the extracellular breakdown of adenine nucleotides released from subcutaneous fibroblasts given to the fact that (1) remodeling of tissue fibroblasts has been noted in response to mechanical acupuncture [57], and that (2) fibroblasts possess the required machinery (e.g., connexin-43 and/or pannexin-1 hemichannels) to release purines under multiple stressful conditions [7,8]. Thus, we believe that, although the interplay between connective tissue manipulation, inflammation, and remodeling *vis a vis* pronociceptive overactivation is rather complex, purines (in particular adenosine) might play relevant modulatory roles in the pathophysiology of chronic myofascial pain. In this regard, parallel overexpression of A_{2A} receptor and ecto-5′-nucleotidase/CD73 was recently associated with transition from acute to chronic neuroinflammatory conditions [58].

Despite the novelty of the information provided in this study, which was designed to investigate the role of endogenously generated adenosine in primary cultures of HSCF, there are certain limitations deserving investigation in the near future. First, in this study, HSCF cells growth and collagen production were investigated under basal (non-stimulated) conditions; it is expected that the fine-tuning control of HSCF cells function by A_{2A} and A_3 receptors might change in cells stimulated with TGF-β and pro-inflammatory mediators (e.g., bradykinin, histamine, IL-1β, TNF-α). Second, no animal/tissue model of wound healing, dermal fibrosis, and myofascial pain was presented; discussion regarding these issues was fully based on data from the literature using uneven experimental approaches. Third, besides the use of exogenously added ADA, no attempts have been made thus far to simulate the subcutaneous microenvironment resulting from infiltration by ADA-bearing inflammatory cells in the vicinity of HSCF. Fourth, repercussions of endogenous adenosine production by HSCF on subcutaneous vasculature and afferent nerve fibers also remain to be elucidated in more integrative tissue models before any firm theory can be reached.

In conclusion, data suggest that the HSCF purinome comprising release sites, ectonucleotidases, and purinoceptors deserves to be explored in the future using multitarget drugs and/or monoclonal antibodies, for instance, against ecto-5′-nucleotidase/CD73 activity applied either alone or in combination with subtype selective adenosine receptor antagonists, in order to show their potential for the treatment of inappropriate wound healing, dermal fibrosis, and myofascial pain (see Figure 6).

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/3/651/s1, Figure S1: Positive identification of adenosine A_1 and A_{2B} receptors on cultured human primary bone marrow stromal cells undergoing osteogenic differentiation.

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Investigation, C.H.-d.-S., A.R.P., D.P.-d.-C., M.A.C., F.F., T.M.-C., S.R., J.P., J.S. and P.C.-d.-S.; Resources, J.S., S.R., P.C.-d.-S.; Data Curation, C.H.-d.-S., A.R.P., D.P.-d.-C., M.A.C., F.F., T.M.-C.; Writing—Original Draft Preparation, C.H.-d.-S., A.R.P., J.S., P.C.-d.-S.; Writing—Review & Editing, C.H.-d.-S., A.R.P., D.P.-d.-C., M.A.C., F.F., T.M.-C., S.R., J.P., J.S. and P.C.-d.-S.; Supervision, M.A.C., J.S., P.C.-d.-S.; Project Administration, P.C.-d.-S.; Funding Acquisition, J.S., P.C.-d.-S. All authors have read and agreed to the published version of the manuscript.

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PAPER 2:

Adenosine A_{2A} receptor-induced overexpression of pannexin-1 hemichannels indirectly mediates fibrosis of the human subcutaneous tissue by favouring ATP release

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Adenosine A_{2A} receptor-induced overexpression of pannexin-1 hemichannels indirectly mediates fibrosis of the human subcutaneous tissue by favouring ATP release

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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 activation (A_{2A}R). The fibrogenic effect of adenosine mimicked that of inflammatory mediators (e.g. histamine, bradykinin), which indirectly favour the release of ATP via pannexin-1 (Panx1) and/or connexin-43 (Cx43) containing hemichannels. Here, we investigated the putative interplay between A_{2A} receptors activation and the expression and function of ATP-releasing Panx-1- and Cx43-containing hemichannels in primary cultures of HSCF. Pre-incubation with two enzymatically-stable A_{2A}R agonists, NECA and CGS21680C, increased (p<0.05) Panx-1 immunoreactivity in HSCF, while the opposite was observed regarding the expression of Cx43-containing hemichannels. Likewise, HSCF treated with NECA and CGS21680 for 7 days spontaneously release higher amounts of ATP compared to non-treated cells. Involvement of the A_{2A}R on collagen production, dual modulation of Panx1 vs. Cx43 expression and ATP release by HSCF was confirmed by blocking the effects of NECA and CGS21680 with the selective A_{2A}R antagonist, SCH442416. Likewise, inhibition of Panx1 hemichannel with probenecid decreased ATP release by HSCF treated with the two A_{2A}R agonists. Overexpression of ATP-releasing Panx1 hemichannels triggered by A_{2A}R agonists contribute to sustain intracellular $[Ca^{2+}]_i$ accumulation when HSCF were challenged with inflammatory mediators, like histamine. We show here for the first time that adenosine acting via A_{2A}R promotes overexpression of Panx1-containing hemichannels, which by indirectly increasing extracellular ATP accumulation generates a vicious cycle to favour subcutaneous tissue fibrosis in 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 (Correa-Gallegos et al. 2019; Cronstein 2011a; Sawynok 2016). 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 and with neighbouring cells by releasing signalling mediators, like ATP among other danger molecules, in response to noxious mechanical stimulation and neuroendocrine and/or inflammatorymediators (Langevin et al. 2004; Pinheirot al. 2013b).

ATP-releasing hemichannels containing connexin-43 (Cx43) or pannexin-1 (Panx1) afford tuned communication between cytosol and the extracellular environment of functionally active fibroblasts, but only those containing Cx43 integrates the macromolecular protein scaffold of GJ communication pores (Willebrords et al. 2017). The two hemichannel subtypes are also differently regulated by phosphorylation, plasma membrane potential and the extracellular concentration of calcium and magnesium ions; unlike Panx1-containing hemichannels, those build-up with Cx43 are normally silent at physiological Ca^{2+} concentrations (e.g. 1.8 mM), thus requiring low external Ca^{2+} conditions to become operative (Fasciani et al. 2013). Apart from its role in intercellular communication, the Cx43 carboxy tail 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) (Zhang et al. 2021).

The cellular amounts of Cx43- and Panx1-containing hemichannels undergo substantial modifications under pathological conditions, which might have significant impact in intercellular communication (Wang, Lincoln, et al. 2007; Morioka et al. 2019; Mori et al. 2006; Willebrords et al. 2017; Chekeni et al. 2010; Laird et al. 2018). Downregulation of Cx43 has been associated with improvements in skin wound healing (Mori et al. 2006; Wang, Lincoln, et al. 2007), while its overexpression is implicated in chronic pain (Morioka et al. 2019; Chen et al. 2012) and delayed healing subsequent to inflammatory conditions (Li et al. 2018) 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 (Yeung et al. 2020; Pinheiro et al. 2013b). Panx1 also plays a role in the development and maintenance of chronic mechanical hypersensitivity (Weaver et al. 2017), as well as in dermal fibrosis (Feig et al. 2017), most probably by releasing adenine nucleotides in response to inflammatory signal mediators (Pinheiro et al. 2013a; Pinheiro et al. 2013b).

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 (Pinheiro et al. 2013b; Goldman et al. 2013; Langevin et al. 2013; Pinheiro et al. 2013a), very little attention has been given to the effects of their metabolite, adenosine, acting via P1 receptors, reviewed in (Burnstock 2017b) (Perez-Aso et al. 2014). 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 (Pinheiro et al. 2013b; Pinheiro et al. 2013a; Herman-de-Sousa et al. 2020). 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 (Herman-de-Sousa et al. 2020), which may be relevant to normal wound healing (Cronstein 2011a; Chan et al. 2013; Perez-Aso et al. 2012; Yokoyama et al. 2008; Perez-Aso and Mediero 2013). 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 where patients present excessive collagen deposition in the skin and visceral organs (Lazzerini et al. 2012).

Controversy however exists on the mechanism(s) underlying the pro-fibrotic action of adenosine via $A_{2A}R$ activation, reviewed in (Shaikh et al. 2016), mostly because cyclic AMP, its principle effector, exerts paradoxical effects on collagen production via differential sensitivity of protein kinase A (PKA) and exchange protein activated by cyclic adenosine monophosphate (EPAC) o this intracellular messenger (Yokoyama et al. 2008; Perez-Aso, Mediero, et al. 2013; Herman-de-Sousa et al. 2020). 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 expression (Perez-Aso et al. 2014). Downstream interference with the equilibrium between transcription promoters and repressors of matrix-producing genes have additionally been hypothesized. 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 (Chan et al. 2013).

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. 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 subcutaneous tissue homeostasis/remodelling and the putative implications of the nucleoside in myofascial inflammatory conditions.

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 (Pinheiroet al. 2013a; Pinheiroet al. 2013b; Herman-de-Sousa et al. 2020). 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 [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) (Herman-de-Sousa et al. 2020; Pinheiro et al. 2013a; Pinheiro et al. 2013b).

SDS–PAGE and Western blotting

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 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

Viability/proliferation studies included the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Herman-de-Sousa et al. 2020; Pinheiro et al. 2013a). HSCF were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL and cultured in supplemented DMEM. Cell cultures were routinely monitored by phase contrast microscopy and characterized on a weekly basis until culture day 28. The MTT assay consists on the reduction of MTT to a purple formazan reaction product by viable cells. In the last 4 h of each test period, cells were incubated with 0.5 mg/mL of MTTfor 4 h in the conditions referred above. The medium was carefully removed, decanted and the stained product dissolved with DMSO before absorbance (A) determination at 600 nm using a microplate reader spectrometer (Synergy HT, BioTek, Winooski,VT, USA). Results were expressed as A/well.

Total collagen determination

Collagen determination was performed using the Sirius Red staining assay (Tullberg-Reinert et al. 1999) by a multidetection microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), as described elsewhere that stains equally well collagen types I and III (Tullberg-Reinert et al. 1999), which are the two main collagen types existing in the human skin, normally at a ratio of 4:1 (Perez-Aso, Mediero, et al. 2013). HSCF were cultured as described for the viability/proliferation studies. 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 (Tullberg-Reinert et al. 1999). 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) using a multidetection microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA), as described elsewhere (Pinheiro et al. 2013a; Pinheiro et al. 2013b). 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). At the beginning of the experiment, 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 (basal). Samples (100 μl) collected at five different incubation times (0–240 s) were immediately stored at −20 °C (Pinheiro et al. 2013b; Pinheiro et al. 2013a). Bioluminescence assays were performed at room temperature. ATP-dependent changes in extracellular luciferase activity were measured as relative luminescence unit (RLU) values integrated over 20 s photon counting periods.

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

Changes in intracellular $[Ca^{2+}]_i$ were measured 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) . Briefly, HSCF were seeded in flat bottom 96-well plates at a density of 3×10^4 cells/ml. Cells were cultured 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). On the day of the experiment, cells were washed twice with Tyrode's solution and incubated at 37 °C for 30 min with the cell-permeant fluorescent Ca^{2+} indicator, Fluo-4 NW (2.5 μM), in 1X HBSS, 20 mM HEPES and 2.5 mM probenecid. After removal of the fluorophore loading solution, cells were washed again twice, and 150 μl of Tyrode's solution was added per culture well. The 96-well cell plates were then loaded into the microplate reader. Reader control was performed using the BioTek's Gen5™ Data Analysis Software. For the recordings, temperature was maintained at 32 °C, and readings were made with 5 seconds of interval, during approximately 30 minutes, using a tungsten halogen lamp. 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)(Henriksen et al. 2006).

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 prepared in dimethyl sulfoxide (DMSO). All other drugs were prepared in distilled water. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of DMSO at the maximal concentration used (0.05% v/v), were observed. The pH of the solutions did not change by the addition of the drugs in the maximum concentrations applied to the preparations. ATP bioluminescence assay kit HS II was from Roche Applied Science (Penzberg, Germany). Bicinchoninic acid (BCA) Protein Assay Kit was from Pierce. Fluo-4 NW dye was supplied by Molecular Probes, Invitrogen, (Waltham, MA, USA). Tissue culture plates: 96-well plates were purchased from Corning; chamber slides were from Nunc (Roskilde, Denmark).

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.

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 vimentin, the intermediate protein filament considered a reliable fibroblast-cell marker (Figure 1 Ai, and Aii).

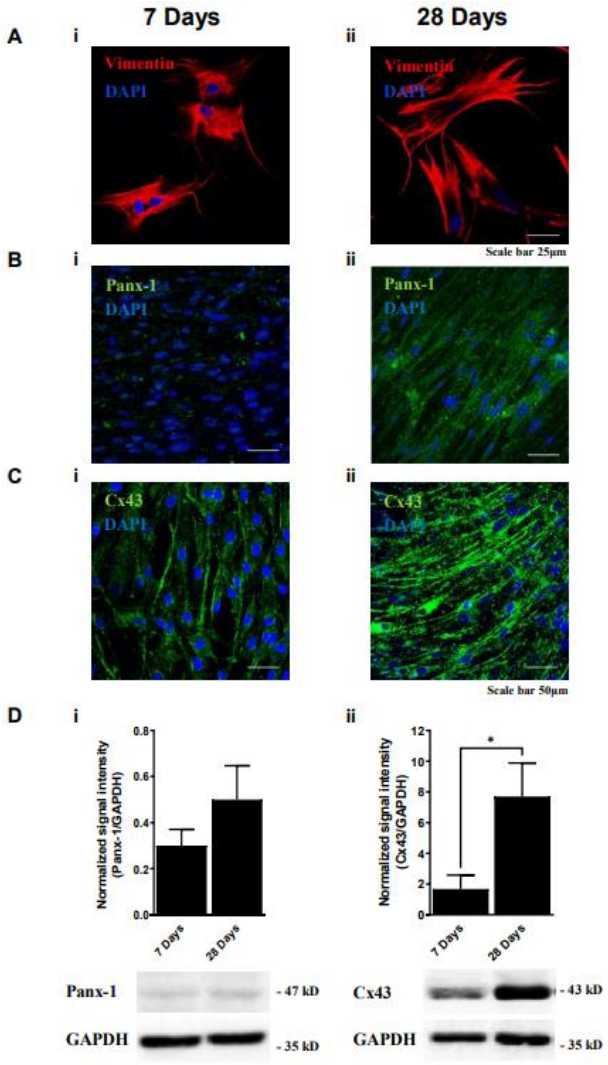


Figure 1. Human subcutaneous fibroblasts (HSCF) exhibit increasing amounts of Panx1 and Cx43 proteins along the time of the cells in culture. Shown are confocal micrographs of HSCF stained against (A) vimentin, (B) Panx1 and (C) Cx43 obtained at culture days 7 (i) and 28 (ii). Nuclei are stained in blue 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: 25 μm (in A) and 50 μm (in B and C). 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.

In a previous study, 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) (Herman-de-Sousa et al. 2020). By immunofluorescence confocal microscopy and Western blot analysis, we showed that HSCF express Panx1 (~48 kDa) and Cx43 (43 kDa) proteins (*cf.* (Pinheiroet al. 2013a; Pinheiroet al. 2013b), which content increases with the time and myofibroblast 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 (Herman-de-Sousa et al. 2020). 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).

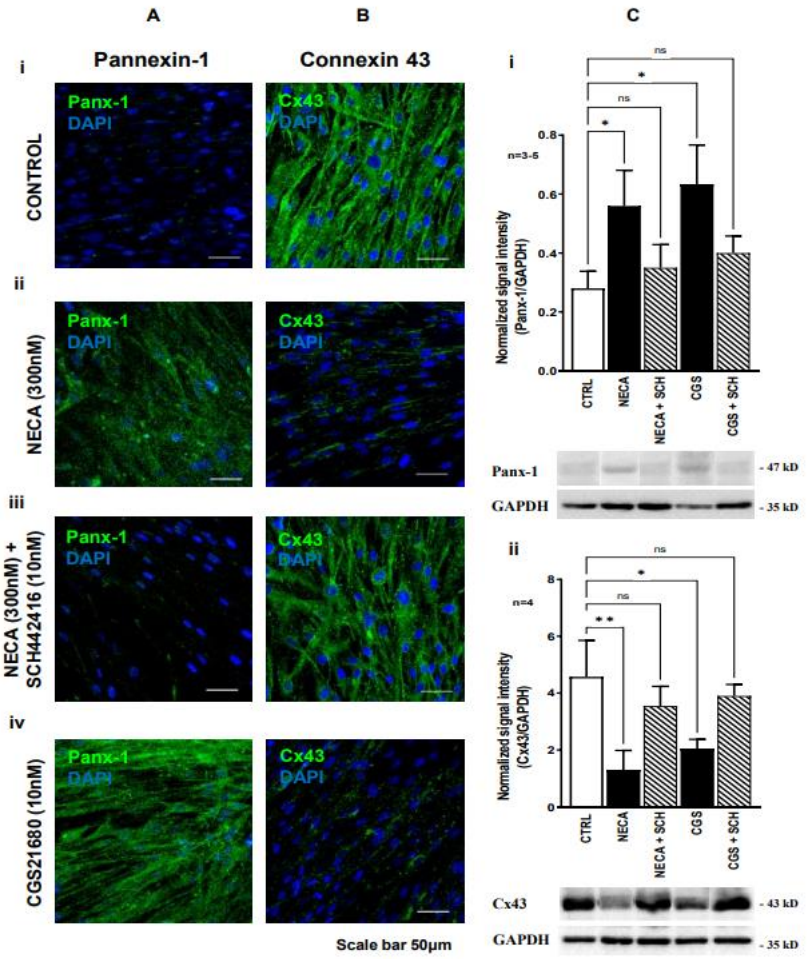


Figure 2. Adenosine A_{2A} receptor activation differently affects the expression of Panx1 and Cx43 in human subcutaneous fibroblasts (HSCF). Panels A and B, show the immunoreactivity against Panx1 and Cx43, respectively, exhibited by HSCF cultured for 7 days either in the absence (i) or in the presence of NECA (300 nM, ii) or CGS21680 (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.

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 (Silverman et al. 2008a). 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).

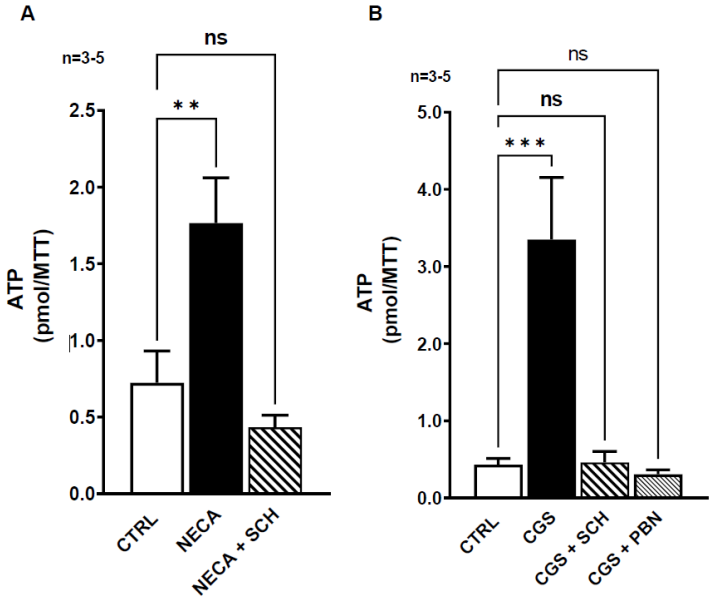


Figure 3. Adenosine A_{2A} receptor activation favours the release of ATP via Panx1-containing hemichannels in cultured human subcutaneous fibroblasts (HSCF). Panels A and B, show the amount of ATP in the culture medium normalized by the number of viable cells (MTT value) per well when HSCF were cultured 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.

Blockage of ATP-releasing Panx1 hemichannels prevents the pro-fibrotic 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 (Ahsan et al. 2014). The pro-fibrotic 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 (Herman-de-Sousa et al. 2020). 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 HSCF only from culture day 21 onwards without affecting cells growth/viability (MTT assay; n=4, data not shown). 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 (Herman-de-Sousa et al. 2020). This hypothesis is strengthened by our results showing that selective inhibition of AC (withSQ22536; 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).

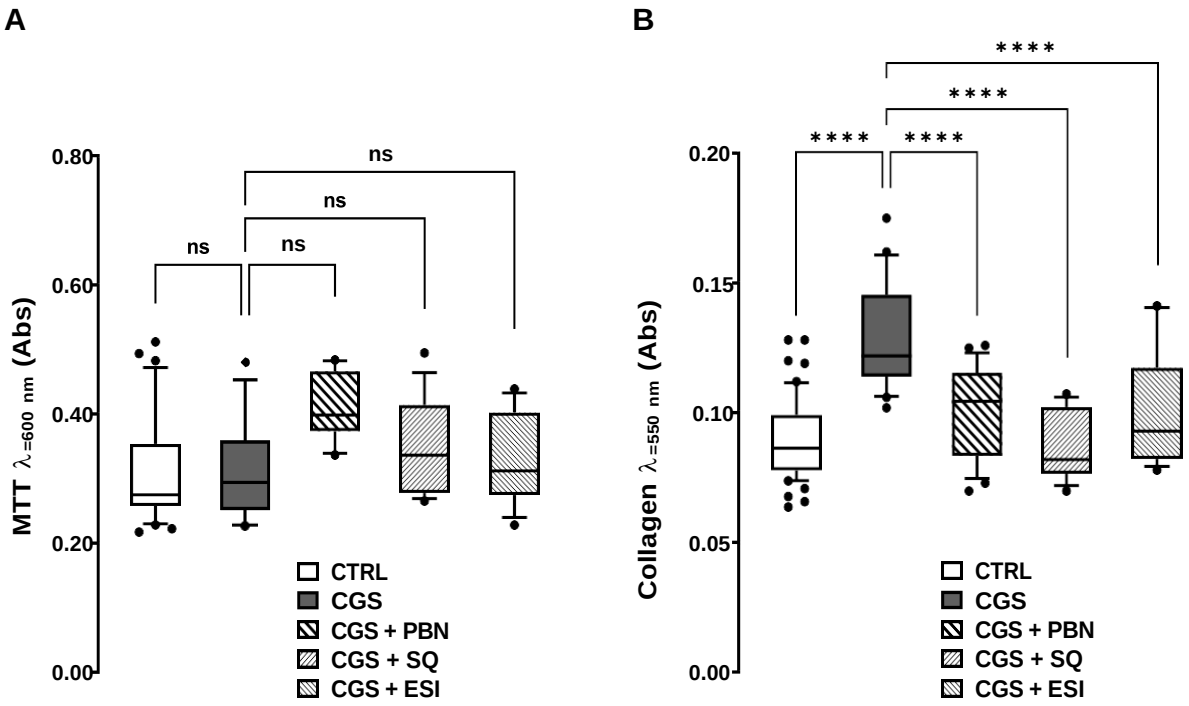


Figure 4. Blockage of ATP-releasing Panx1 hemichannels prevents the pro-fibrotic effect of adenosine via A_{2A} receptor activation on 7-day cultures of human subcutaneous fibroblasts (HSCF). The adenosine A_{2A} receptor agonist, CGS21680 (10 nM), increases collagen production (Sirius Red staining, 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.

Adenosine A_{2A} receptor-induced Panx1 overexpression contributes to sustain histamine-induced $[Ca^{2+}]_i$ accumulation inside human subcutaneous fibroblasts (HSCF).

Taking into consideration that 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 (Pinheiro et al. 2013b), 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.* (Pinheiro et al. 2013b).

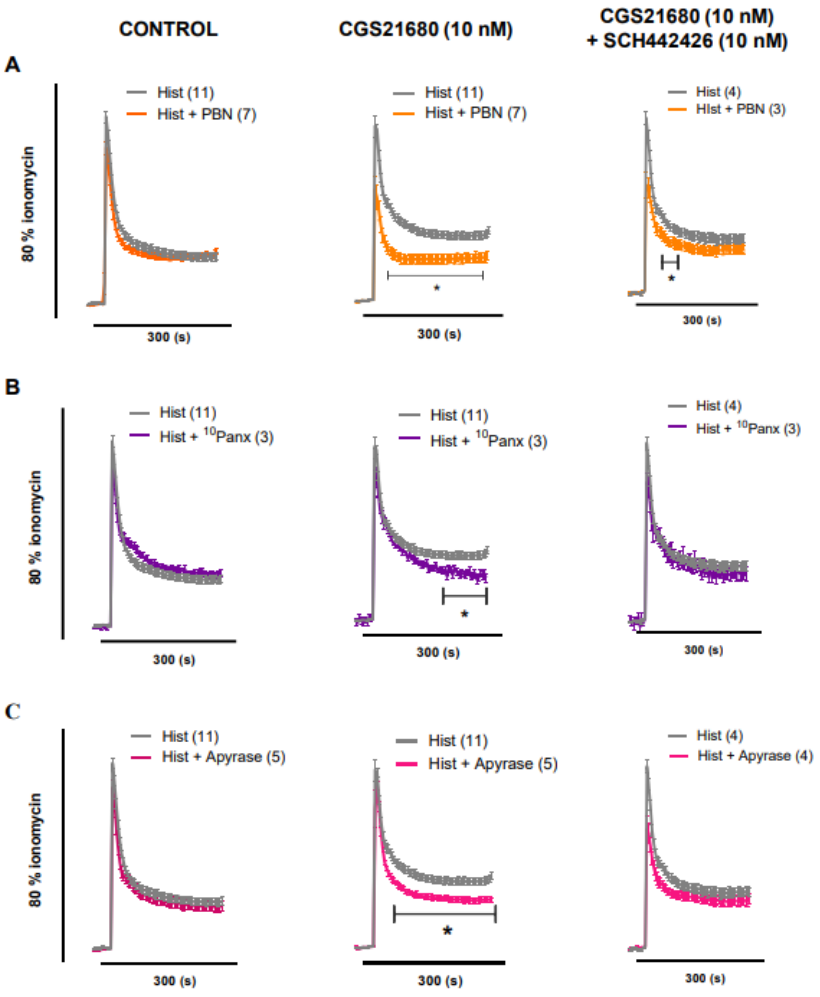


Figure 5. Adenosine A_{2A} receptor-induced Panx1 overexpression contributes to sustain intracellular $[Ca^{2+}]_i$ accumulation caused by histamine (Hist) in human subcutaneous fibroblasts (HSCF). Shown are fluorescence $[Ca^{2+}]_i$ transients subsequent to Hist (100 μM) application in HSCF grown in culture for 7 days eitherin 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.

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). Despite 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)(Wang, Ma, et al. 2007), 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 (Pinheiroet al. 2013b).

Discussion

Considering that fascia is a mechano-sensitive organ bridging all parts of the body which is composed mainly by fibroblasts interacting with large number of neurons mediating proprioception and nociception (Bordoni et al. 2015; Kumka et al. 2012), one can easily understand that disorganization of this complex network structure may affect health condition in many different ways (Adstrum et al. 2017b). Therefore, we foresee that investigating the molecular mechanisms underlying fascial fibroblasts central signalling role might contribute to unravel alterations of the whole body system in myofascial syndromes, likefibromyalgia.

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 fosters the release of large amounts of ATP by 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 (like 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 (Li et al. 2018; Chen et al. 2012; Morioka et al. 2019; Weaver et al. 2017). 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 (Penuela et al. 2013; Pinheiro et al. 2013b). Panx-1 hemichannels have also been increasingly linked to inflammation (Kameritsch et al. 2020), as they integrate inflammasome activation and promote secretion of pro-inflammatory mediators, like interleukin-1β and histamine (Yeung et al. 2020; Pinheiro et al. 2013b). The launch of ATP release via Panx-1 hemichannels is additionally involved in neutrophil chemotaxis (Bao et al. 2013). 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 (Bao et al. 2013). Likewise, the release of ATP via Panx1 hemichannels also plays a role in the development and maintenance of chronic mechanical hypersensitivity (Weaver et al. 2017) and in dermal fibrosis (Feig et al. 2017).

Notwithstanding the fact that (1) adenosine A_{2A} receptors activation simulates the pro-fibrotic action of inflammatory mediators (e.g. histamine, bradykinin), which indirectly favour ATP release by Panx1 hemichannels (Pinheiro et al. 2013b; Pinheiro et al. 2013a), and that (2) adenosine resulting from the breakdown of released ATP extensively accumulates in the extracellular milieu thus favouring activation of A_{2A} receptors located in close proximity to the adenosine forming enzyme, ecto-5'-nucleotidase/CD73 (Herman-de-Sousa et al. 2020), 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 increasing collagen synthesis, inflammation resolution and wound healing (Cronstein 2011a; Herman-de-Sousa et al. 2020) Coincidently, Panx1 expression and function in HSCF paralleled the amounts of adenosine-forming enzyme, ecto-5'-nucleotidase, and the A_{2A} receptor in these cells along culture maturation (Herman-de-Sousa et al. 2020). 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 multifunctionalglycoprotein CD26 which exhibits dipeptidylpeptidase IV (DPP4) activity. Thus, mononuclear inflammatory cell infiltrates carrying high surface amounts of CD26–ADA complexes may negatively impact on adenosine mediated dermal nociception (vianeuronal A_1 receptors) and wound healing (via fibroblast A_{2A}) (Guieu et al. 2012), while increasing inosine-sensitive A_3-mediatedactions in fibromyalgia patients (Fais et al. 2013; Herman-de-Sousa et al. 2020).

On the other hand, it has been shown that fibroblasts overexpressing adenosine A_{2A} receptors may produce a hostile environment leading to dermal fibrosis (Feig et al. 2017) and scleroderma (Lazzerini et al. 2012). Likewise, upon tissue destruction by ionizing radiation extracellular adenosine accumulation may promote skin fibrosis and scarring via A_{2A} receptors activation (Perez-Aso et al. 2016). 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 (Pinheiro et al. 2013a; Pinheiro et al. 2013b); see also (Cronstein et al. 2017; Chan et al. 2006). In a recent paper, (Yang et al. 2020) implicated Panx1 overexpression in a P2X7-independent inflammatory mechanism undertaken by activation of the TNF-α/IL-1β pathway in human umbilical vein endothelial cells, 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 (Harcha et al. 2019). 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 (Flores-Muñoz et al. 2021).

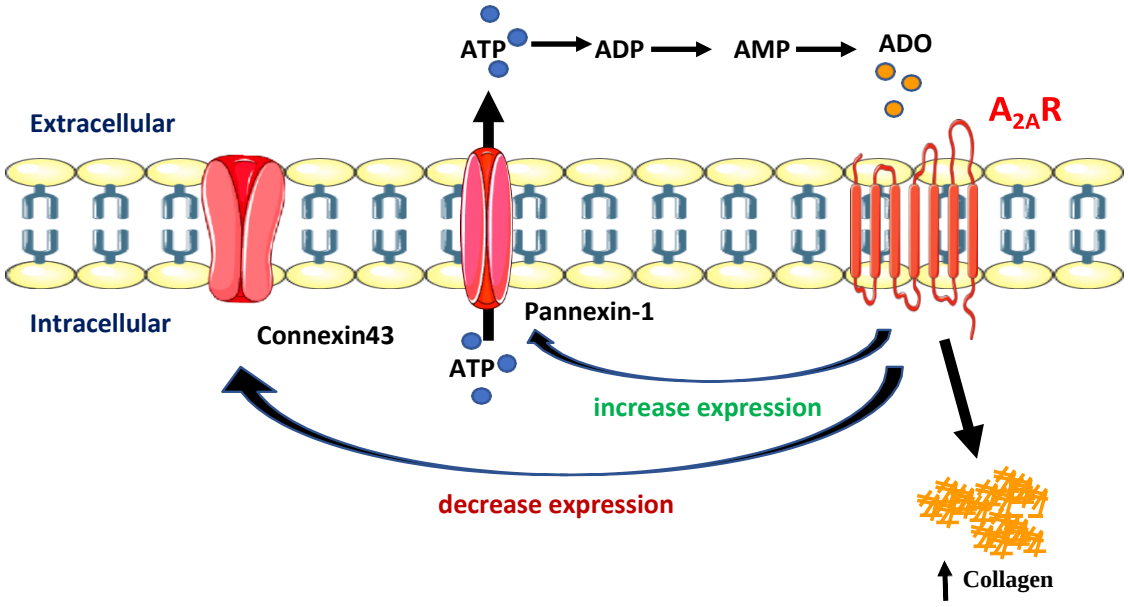


Figure 6. Adenosine A_{2A} receptors activation exerts a dual role in the expression of Panx1- and Cx43-containing hemichannels with significant functional repercussions on ATP release, histamine-induced $[Ca^{2+}]_i$ mobilization and collagen production by HSCF, which might be critical to the pathogenesis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

Contrariwise, Cx43 levels normally decay one day after skin injury returning to homeostatic levels after wound closure, both in humans and rodents (Lampe et al. 1998). 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 (Laird et al. 2018). 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 (Rhett et al. 2011). Moreover, acute downregulation of Cx43 at wounded sites tapers inflammatory responses and increases fibroblasts migration and keratinocytes proliferation (Mori et al. 2006). Downmodulation of the expression and function of Cx43 may play a role against chronic neuropathic pain (Morioka et al. 2019), in particular when this protein is overexpressed (Chen et al. 2012).

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) (Fasciani et al. 2013). 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. (Li et al. 2016), 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 pro-fibrotic 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 (Adstrum et al. 2017b).

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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

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CHAPTER 3. DISCUSSION AND CONCLUSIONS

Fascia is considered our largest sensory organ; it is densely innervated and plays relevant roles in scarring, tissue remodeling, inflammation and pain perception (Correa-Gallegos et al. 2019; Cronstein 2011a; Sawynok 2016; Fede et al. 2020; Fede et al. 2021). Recently, it has been suggested that endogenously released purines, namely ATP and adenosine, may putatively play a role in fascia pathophysiology, through the activation of both P2 and P1 purinoceptors, respectively (Burnstock 2017a; Perez-Aso et al. 2014; Butts et al. 2016). Fibroblasts, the main cellular constituents of fascial networking, convey long distance information throughout the body via intercellular syncytial communication, along with paracrine interactions with cells in the vicinity, like sensitive nerve endings and immunocytes In adulthood, fibroblasts are relatively quiescent unless tissue repair mechanisms or dynamic structural changes are initiated (Plikus et al. 2021). To facilitate tissue repair, signaling and physical factors induce quiescent fibroblasts to form myofibroblasts, which, through their expression of contractile proteins such as α-SMA, orchestrate biomechanical remodeling and contraction via traction force. Although, traditionally, myofibroblasts were thought to be terminally differentiated, accumulating evidence indicates that they are, in fact, temporary and reversible.

The last several decades have seen remarkable progress in our understanding of fibroblast biology across organs and conditions (Abbasi et al. 2020). As our appreciation of essential fibroblast functions extends well beyond ECM synthesis, so does the excitement over novel therapeutic possibilities that modulating them *in situ* can bring for a broad spectrum of diseases characterized by aging, pathologic remodeling, and tissue fibrosis. Emerging areas of investigation, including cross-regulation between wound fibroblasts, immune cells, and the peripheral nervous system, will be critical for understanding how to restore tissues to their pre-injury state (Plikus et al. 2021; Abbasi et al. 2020).

Despite adenosine has been increasingly involved as a signaling molecule in fascial remodeling, there are still gaps in our knowledge regarding the molecular mechanisms by which it affects homeostasis / remodeling of the human fascia, as well as about the participation of the nucleoside in chronic myofascial pain syndromes, like fibromyalgia. Here, we used primary fibroblast cultures of the human subcutaneous tissue (HSCFs) as a model to study the mechanisms underlying the purinergic signaling role in cells growth and remodeling, intracellular calcium handling and response to inflammatory mediators, focusing on the actions of the ATP metabolite, adenosine.

HSCFs exhibit strong immunoreactivity against A_{2A} and A_3 adenosine receptor subtypes, with only minute amounts of A_{2B} and A_1 receptors. Interestingly, HSCFs in culture accumulate high amounts of adenosine in the extracellular milieu when ATP, ADP and AMP were used as substrates (this study; see also Pinheiro et al. 2013a; Pinheiroet al. 2013b). These findings suggest that HSCFs are endowed with enzymes of the ecto-nucleotidase cascade (e.g. NTPDase1 and NPPs) responsible for the hydrolysis of ATP directly into AMP, which is subsequently dephosphorylated into adenosine by ecto-5´nucleotidase / CD73. The extracellular accumulation of adenosine is only possible if the nucleoside inactivation mechanisms, both cellular uptake and extracellular deamination by ADA (Cronstein et al. 2017) are relatively inactive, as we showed here. Thus, adenosine accumulation in the extracellular medium predicts an outstanding effect of the most expressed adenosine A_{2A} (pro-fibrotic) and A_3 (anti-fibrotic) receptor subtypes in HSCFs, which happened to be proved in this study.

Mounting evidence suggest that adenosine regulates inflammation, fibrosis and pain in several organs through different mechanisms associated with distinct patterns of adenosine receptors expression and activation profiles (Cronstein et al. 2017), but their involvement in pathological conditions is still uncertain. Our data show that adenosine

A_{2A} receptor activation favors collagen production by HSCFs. This is in line with fact that overexpression of adenosine A_{2A} receptor has been detected in fibroblasts of scleroderma patients leading to excessive collagen deposition in the skin (Lazzerini et al. 2012). Likewise, pharmacological blockade of the A_{2A} receptor prevented radiation- induced dermal fibrosis in a murine model, revealed as decreases of skin thickness, collagen content and tissue misalignment (Perez-Aso et al. 2016; Velickovic et al. 2019).In addition to the anti-fibrotic effect of the A_{2A} receptor, it also plays an important anti- inflammatory role by (i) inhibiting neutrophil adhesion and recruitment, (ii) decreasing the macrophage expression of cytokines, (iii) preserving the integrity of the endothelial cells barrier, and (iv) preventing edema (Williams 2011).

On the other hand, purines (like adenosine and inosine) may be involved in pain perception of fibromyalgia patients (Fais et al. 2013). High levels of inosine, hypoxanthine and xanthine were detected in sera of fibromyalgia patients, while the opposite was observed for adenosine (Fais et al. 2013), mostly because measurements were made without restraining superfluous ADA activity observed in these patients. Thus, under these circumstances, the unforeseen breakdown of adenosine into inosine and the subsequent activation of the A_3 receptor in fibroblasts localized in the vicinity of ADA-transporting inflammatory cell infiltrates might favor deficient connective tissue remodeling and failure in maintaining its viscoelastic properties. Notwithstanding this, harnessing the endogenous anti-nociceptive A_3 receptor pathway produces effective pain relief without altering the normal protective nociception and without producing reward effects associated with abuse potential in ongoing phase II/III clinical trials for pain-free conditions (Janes et al. 2016). Moreover, a recent study demonstrated that activation of A_3 receptors restores morphine anti-nociception in opioid-tolerant patients (Leduc-Pessah et al. 2021). In view of these controversies, a word of caution is warranted before making

precipitating conclusions taking into account that adenosine receptor subtypes amounts and distribution between different tissues may be decisive to understand their overall participation in a given pathophysiology condition, particularly when a global vision of the whole scenario is not yet provided.

Hemichannels containing Panx-1 and Cx43 are widely accepted as putative mediators of ATP translocation to the extracellular milieu in non-excitable cells, including fibroblasts (Penuela et al. 2013; Pinheiro et al. 2013a; Pinheiro et al. 2013b). Interestingly, changes in the expression and activity of Panx-1 and Cx43 have been reported in disease conditions involving fibrosis, inflammation and pain (Li et al. 2018; Chen et al. 2012; Morioka et al. 2019; Weaver et al. 2017). Panx-1 and Cx43 have opposing roles in dermal healing and fibrosis. It has been demonstrated that inhibition of Panx-1-mediated ATP release prevents skin fibrosis by downregulating extracellular adenosine formation (Feig et al. 2017). On the other hand, Cx43 levels normally decay one day after skin injury returning to homeostatic levels after wound closure, both in humans and rodents (Lampe et al. 1998). Taking this into account, 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 favor recovery of dermal histoarchitecture and skin mechanical resistance (Laird et al. 2018). Panx1 is also important for the inflammasome formation and the secretion of pro-inflammatory mediators, such as interleukin-1β or histamine (Yeung et al. 2020; Pinheiro et al. 2013b). Acute downregulation of Cx43 at wounded sites tapers inflammatory responses and increases fibroblasts migration and keratinocytes proliferation (Mori et al. 2006). In the context of pain, Panx1 plays an important role in the development and maintenance of neuropathic pain after nerve damage and should be considered as a practicable therapeutic target for

this often-intractable condition. Pharmacological inhibition of Panx1 alleviated mechanical hypersensitivity when drugs were administered systemically (Weaver et al. 2017). Accordingly, recent studies have shown that connexins may be crucial in the induction and maintenance of chronic pain, and modulation of connexins activity or expression ameliorates nociceptive hypersensitivity in multiple chronic pain models. (Morioka et al. 2019) (Figure 3).

This overview of the role of Panx-1 and Cx43 in pathological conditions emphasizes the putative therapeutic relevance of manipulating the expression and function of these two hemichannel constituents in fibrosis, inflammation and pain. Data show here for the first time that adenosine, via A_{2A} receptors activation, significantly increases the expression and function of Panx-1 in HSCFs, while causing the opposite effect in what concerns Cx43. These findings contribute to elucidate the mechanisms underlying the pro-fibrotic effect of the nucleoside in the pathogenesis of dermal malignancies associated with cellular overexpression of the A_{2A} receptor. It seems that accumulation of adenosine is part of a self-regenerating pathway consisting in (1) the activation of A_{2A} receptors, leading to (2) Panx-1 hemichannels overexpression, and (3) ATP release facilitation. Increases in extracellular ATP amounts mediate autocrine and paracrine actions on HSCFs either directly, via P2 purinoceptors activation (Pinheiro et al. 2013a; Pinheiro et al. 2013b), or indirectly, via P1 (namely A_{2A}) receptors after the extracellular nucleotide breakdown to adenosine, which may contribute to increase the sensitivity of Ca^{2+}-mediated signaling events triggered by inflammatory mediators, like histamine.

Thus, we anticipate that perturbation of adenosine homeostasis favors changes in connective tissue wound healing and remodeling, response to inflammatory mediators, and pain perception, which via the fascial networking may lead to devastating

repercussions throughout the body (Adstrum et al. 2017a). Trigger points submitted to increased tissue tension (due to inflammation and/or architectural distortion) may cause prolonged release of adenine nucleotides and adenosine formation, which might sustain chemical responses throughout the connective tissue network, further restraining its compliance globally. This occurrence could, eventually, be the basis for deficient peripheral modulation of pathogenic inputs that may be involved in nociceptive sensitization and, ultimately, chronic pain conditions.

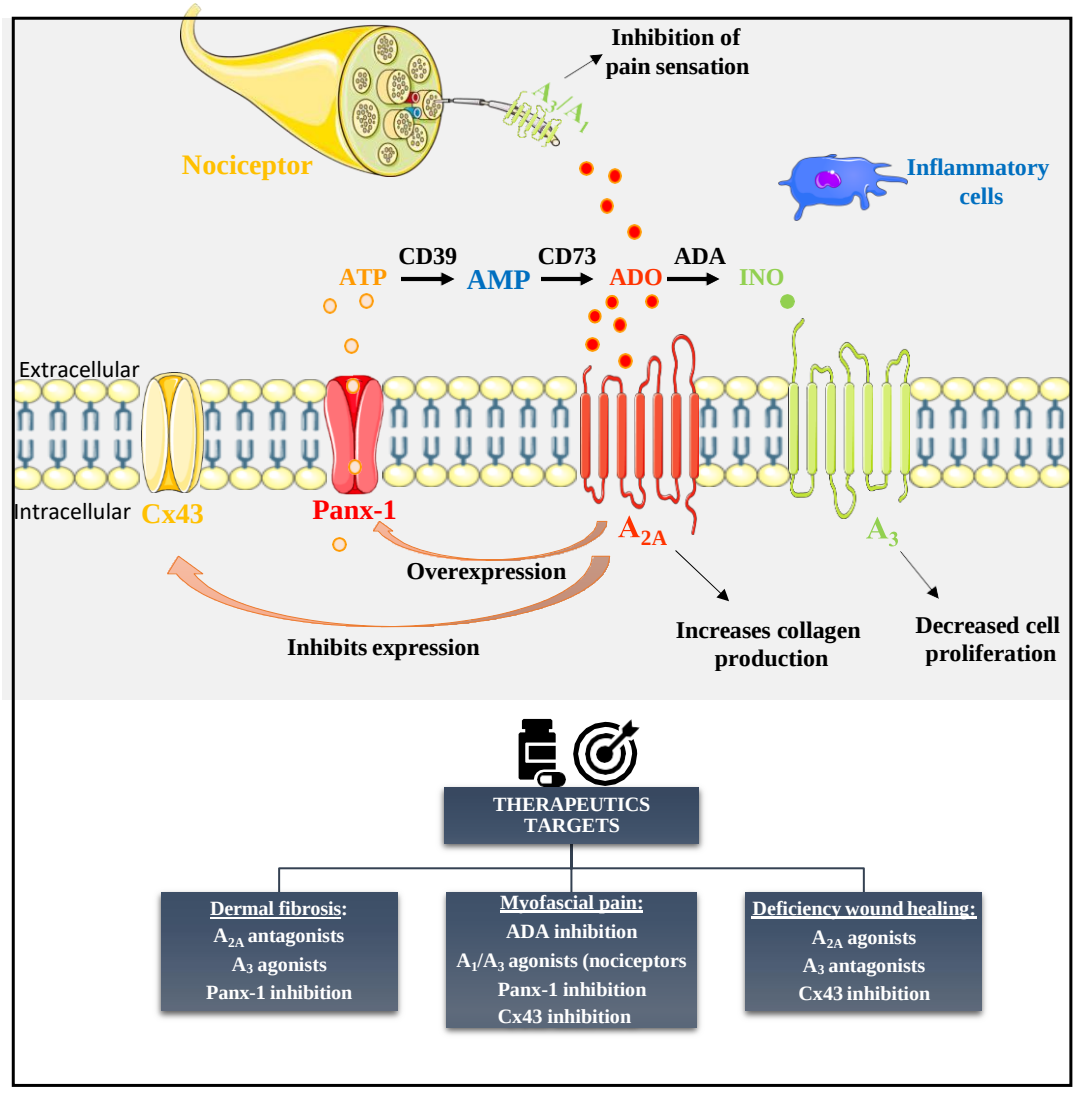


Figure 3: Therapeutic implications of purinergic signaling targets in human subcutaneous fibroblasts.

In conclusion, data suggests that the purinergic signaling cascade in human subcutaneous fibroblasts and more broadly in fascia, deserves to be further explored in the future to design novel drug targets for the treatment of inflammatory, fibrotic and painful myofascial disease conditions. In this sense, considering that overexpression of the A_{2A} receptors is a common feature in fibroblast malignancies (eg, dermal fibrosis, scleroderma, dermal radiation injury), association between A_{2A} receptor antagonists and A_3 receptor agonists may be proposed. On the other hand, activation of the A_{2A} adenosine receptor can be a pharmacological target to accelerate wound healing in patients with diabetes or other pathologies associated with impaired dermal healing. Moreover, rebalancing the extracellular adenosine/inosine concentrations using ADA blockers may be useful to promote immune suppression and nociceptive relief in myofascial pain conditions. The use of adenosine A_3 receptor agonists seems to be promising for the treatment of myofascial pain syndromes, such as fibromyalgia. Finally, the development of more selective pharmacological tools with better pharmacokinetic profiles will certainly contribute to translate Panx-1 and Cx43 hemichannel inhibitors to clinical trials for the treatment of painful myofascial syndromes.

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