Adenosine exerts a pro-fibrotic and pro-inflammatory effect on rat subcutaneous fibroblasts via A_{2A} receptors activation

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Resumo

Descobertas recentes sugerem que o tecido celular subcutâneo pode sofrer inflamação crónica e consequente fibrose do tipo miofascial comprometendo a resposta fisiológica deste tecido a estímulos mecânicos e/ou inflamatórios. Tendo em conta que o tecido conjuntivo subcutâneo possui elevada quantidade de recetores da dor e fibras nociceptivas, facilmente se pode especular que a alteração das suas propriedades pode alterar significativamente a perceção dolorosa subjacente a episódios de dor miofascial.

Os nucleótidos e nucleósidos da adenina, designadamente o adenosina 5'-trifosfato (ATP) e o seu metabolito adenosina (ADO), são importantes moduladores da atividade nervosa e da resposta inflamatória/imune. Os seus níveis aumentam drasticamente no meio extracelular em situações de stresse celular, tal como fenómenos de isquemia/reperfusão, durante a resposta inflamatória e após estimulação mecânica.

Especificamente, o nosso grupo de trabalho demonstrou que os fibroblastos do tecido celular subcutâneo humano respondem a estímulos mecânicos e mediadores inflamatórios, como por exemplo a bradicinina (1) e a histamina (2), através da libertação massiva de ATP. Uma vez no espaço extracelular, o ATP é rapidamente hidrolisado em adenosina monofosfato (AMP) por NTPDases 1 e 2 e, subsequentemente, em ADO pela ecto-5'-nucleotidase (Ecto-5'-NT). Curiosamente, a ADO tem tendência a acumular-se na biofase em virtude destas células possuírem mecanismos de inativação, recaptação celular e desaminação pela adenosina desaminase (ADA) pouco eficientes. Este aspeto particular característico das células mesenquimatosas favorece a possibilidade de ativação dos recetores A_{2A} ($A_{2A}R$) e A_3 (A_3R) sensíveis à ADO, que se encontram abundantemente expressos fibroblastos subcutâneos (3).

Contudo, existe ainda alguma controvérsia em torno do papel da ADO como molécula sinalizadora na remodelação do tecido celular subcutâneo, nomeadamente em contextos de fibrose dérmica e/ou dor miofascial. Por questões de facilidade de obtenção e utilização experimental, neste trabalho estudou-se o papel de análogos estáveis da ADO na viabilidade/proliferação (ensaio MTT) e na produção de colagénio (ensaio Sirius Red) em culturas de fibroblastos subcutâneos de ratazana na ausência e na presença de histamina, um conhecido agente pró-inflamatório. Paralelamente, foram realizados ensaios para estudar a sinalização intracelular mediada pelo cálcio (por sonda fluorescente) e a libertação de ATP (por bioluminescência).

À semelhança do que que foi observado nos fibroblastos subcutâneos humanos, as células de ratazana também expressam em maior quantidade $A_{2A}R$ e A_3R, exibindo apenas quantidades residuais de recetores dos subtipos A_1 (A_1R) e A_{2B} ($A_{2B}R$).

O agonista seletivo dos A_3R, 2-Cl-IB-MECA, aumentou a proliferação celular sem modificar significativamente a produção de colagénio; o efeito pró-fibrótico da 2-Cl-IB-MECA (1 μM) atingiu significado estatístico ao fim de 21 dias de incubação ($p<0.05$), tendo sido o mesmo resgatado na presença do antagonista dos A_3R, MRS1191 (10 nM, $p>0.05$). A ativação seletiva dos $A_{2A}R$ com CGS21680 aumentou de forma dependente da concentração tanto a proliferação celular como a deposição de colagénio nas culturas fibroblastos subcutâneos de ratazana. O efeito pró-fibrótico do CGS21680 (30 nM) também foi mais marcado ao 21º dia de cultura e o mesmo foi significativamente atenuado pelo bloqueio seletivo dos $A_{2A}R$ com SCH442416 (10 nM).

De modo análogo ao verificado em culturas de fibroblastos do tecido subcutâneo humano, a histamina (100 μM) promoveu o crescimento/viabilidade celular, aumentou os níveis intracelulares de $[Ca^{2+}]_i$ e a libertação de ATP nos fibroblastos subcutâneos de ratazana. A histamina (100 μM) causou um aumento rápido dos níveis intracelulares de $[Ca^{2+}]_i$ cuja sustentabilidade se deve ao influxo deste ião através da membrana plasmática, já que este efeito foi abolido pela remoção do Ca^{2+} do meio extracelular com adição de EGTA a 100 μM. Contrariamente, a depleção dos reservatórios intracelulares de Ca^{2+} pela tapsigargina (2 μM), um inibidor da Ca^{2+}-ATPase do retículo endoplasmático, não alterou significativamente ($p>0.05$) o efeito da histamina (100 μM) na acumulação $[Ca^{2+}]_i$ pelos fibroblastos subcutâneos de ratazana em cultura.

Considerando que a sustentação dos níveis intracelulares de $[Ca^{2+}]_i$ induzida pela histamina (100 μM) se pode dever à cooperação com o ATP libertado pelas células em resposta ao mediador inflamatório, tal como foi demonstrado em fibroblastos subcutâneos humanos em cultura (2), investigou-se a ação de vários inibidores da libertação de nucleótidos por hemicanais contendo conexinas (Cx) e panexinas (Panx) sobre este fenómeno. Apenas a carbenoxolona (300 μM), um inibidor não-seletivo das conexinas, Cx26, Cx30, Cx32, Cx43, e Cx46, também capaz de bloquear hemicanais contendo Panx1, logrou reduzir progressivamente os níveis intracelulares de $[Ca^{2+}]_i$ após a resposta inicial rápida da histamina (100 μM) nos fibroblastos de ratazana cultivados durante 7 dias. Nenhum dos outros inibidores testados, i.e. mefloquina (3 μM, um inibidor de canais contendo Cx36 e Cx50), ácido flufenâmico (100 μM, bloqueador

preferencial de hemicanais com Cx43), e o péptido inibitório da Panx1, ^{10}Panx (100 μM), se mostraram eficazes nessa tarefa.

Considerando o efeito pró-fibrótico do agonista seletivo para os $A_{2A}R$, resolvemos avaliar o seu papel na libertação de ATP e na acumulação intracelular de $[Ca^{2+}]_i$ promovidas pela histamina nos fibroblastos subcutâneos de ratazana cultivados durante 7 dias com CGS21680 (30 nM). O tratamento das células com o agonista dos $A_{2A}R$ aumentou cerca de 9 vezes o efeito facilitatório da histamina (100 μM) na libertação de ATP a partir dos fibroblastos subcutâneos de ratazana. O efeito potenciador do CGS21680 (30 nM) sobre a libertação de ATP promovido pela histamina foi prevenido na presença do antagonista dos $A_{2A}R$, SCH442416 (10 nM). Do mesmo modo, o bloqueio dos $A_{2A}R$ promoveu o declínio dos níveis intracelulares de $[Ca^{2+}]_i$ após a resposta inicial rápida da histamina (100 μM), de forma análoga à verificada na presença de carbenoxolona (300 μM).

Em conclusão, dos resultados experimentais mostram que o $A_{2A}R$ parece exercer um efeito pró-fibrótico e pró-inflamatório nos fibroblastos subcutâneos de ratazana, à semelhança do que se verifica noutros tecidos incluindo o tecido celular subcutâneo humano. Neste contexto, demonstrou-se pela primeira vez neste trabalho que a ativação sustentada do $A_{2A}R$ da adenosina exerce um efeito sinérgico com a histamina (e, porventura, com outros mediadores inflamatórios) na acumulação intracelular de $[Ca^{2+}]_i$ e na promoção da libertação de ATP (sinalizador da iminência de dano celular). Resta, no entanto, explorar o papel da adenosina, por via dos $A_{2A}R$, (i) nos mecanismos de libertação de ATP, incluindo na expressão e funcionamento dos hemicanais sensíveis à carbenoxolona, e (ii) na interação próxima entre fibroblastos subcutâneos, terminais nervosos sensitivos e células inflamatórias/do sistema imune que invadem o tecido celular subcutâneo em contexto de doença (e.g. fibrose dérmica, fibromialgia e dor miofascial).

Palavras-chave: fibroblastos subcutâneos, adenosina, recetores A_{2A} e A_3, histamina, libertação de ATP, hemicanais, sinalização mediada por cálcio, dor miofascial.

Abstract

Recent findings suggest that fascia can undergo changes secondary to chronic inflammation and consequent myofascial fibrosis, compromising its physiological response to mechanical and/or inflammatory stimuli. Bearing in mind that the subcutaneous connective tissue is richly innervated by pain receptors and nociceptive fibers, one can easily speculate this tissue properties alteration can significantly modify pain perception underlying myofascial pain episodes.

Adenine nucleotides and nucleosides, namely adenosine 5'-triphosphate (ATP) and its metabolite adenosine (ADO), are important modulators of neural activity and inflammatory/immune responses. Their levels drastically increase in the extracellular milieu upon cellular stress, such as ischemia/reperfusion, during inflammatory response and after mechanical stimulation.

Specifically, our group demonstrated that human subcutaneous fibroblasts respond to mechanical stimuli and inflammatory mediators such as bradykinin (1) and histamine (2) through massive ATP release. Once on the extracellular milieu, ATP is promptly hydrolyzed into adenosine monophosphate (AMP) by NTPDases 1 and 2 and, subsequently, into ADO by ecto-5'-nucleotidase (Ecto-5'NT). Curiously, ADO tends to accumulate in the extracellular milieu due to the fact that in these cells the ADO inactivation mechanisms, cellular uptake and deamination by adenosine deaminase (ADA) are deficient. This particular aspect of mesenchymal cells favors the possibility of activation of ADO-sensitive A_{2A} ($A_{2A}R$) and A_3 (A_3R) receptors, which are abundantly expressed in subcutaneous fibroblasts (3).

However, controversy still exists regarding the ADO role as a signaling molecule in subcutaneous tissue remodeling, namely in the context of dermal fibrosis and/or myofascial pain. Due to ease of obtaining and experimental use, this work focused on studying the role of ADO stable analogs on the viability/proliferation (MTT assay) and collagen production (Sirius Red assay) of rat subcutaneous fibroblasts cultures, either in the absence or in the presence of histamine, a known pro-inflammatory agent. In parallel, experiments were conducted to assess intracellular calcium signaling (by fluorescent probe) and ATP release (by bioluminescence) under similar experimental conditions.

Similarly to what has been observed in human subcutaneous fibroblasts, rat fibroblasts also express $A_{2A}R$ and A_3R in greater quantities, displaying minute amounts of the A_1 (A_1R) and A_{2B} ($A_{2B}R$) subtype receptors.

The selective A_3R agonist, 2-Cl-IB-MECA, increased cell proliferation without significantly modifying collagen production; the pro-fibrotic effect of 2-Cl-IB-MECA (1 μM) reached statistical significance after 21 days of incubation ($p<0.05$), having this effect been rescued in the presence of the A_3R antagonist, MRS1191 (10 nM, $p>0.05$). The selective activation of the $A_{2A}R$ with CGS21680 concentration-dependently increased both cell proliferation and collagen deposition in rat subcutaneous fibroblast cultures. The pro-fibrotic effect of CGS21680 (30 nM) was also clearer at the 21st day of culture, being significantly attenuated by selective blockage of the $A_{2A}R$ with SCH442416 (10 nM).

Like that observed in human fibroblast cultures, histamine (100 μM) promoted cell growth/viability, increased intracellular $[Ca^{2+}]_i$ accumulation and ATP release from rat subcutaneous fibroblasts. Histamine (100 μM) caused a rapid rise in intracellular $[Ca^{2+}]_I$, whose maintenance was due to Ca^{2+} influx through the plasma membrane, as this effect was abolished by Ca^{2+} removal from the extracellular medium with the addition of EGTA (100 μM). Contrariwise, depletion of intracellular Ca^{2+} reservoirs with thapsigargin (2 mM), an endoplasmic reticulum Ca^{2+} inhibitor, did not alter significantly ($p>0.05$) histamine (100 μM)-induced $[Ca^{2+}]_i$ accumulation by rat subcutaneous fibroblasts.

Considering that maintenance of high $[Ca^{2+}]_i$ levels induced by histamine (100 μM) may be due to ATP release by fibroblast cells in response to the inflammatory mediator, as demonstrated in human subcutaneous fibroblasts (2), we investigated the action of several modulators of nucleotide releasing hemichannels containing connexins (Cx) and pannexins (Panx). Among these, only carbenoxolone (300 μM), a non-selective inhibitor of connexins Cx26, Cx30, Cx32, Cx43 and Cx46, also capable of blocking Panx1-containing hemichannels, was able to progressively reduce intracellular $[Ca^{2+}]_i$ levels after the fast initial rise produced by histamine (100 μM) on rat subcutaneous fibroblasts cultured for 7 days. None of the other modulators tested, e.g. mefloquine (3 μM, Cx36 and Cx50 inhibitor), flufenamic acid (100 μM, preferentially blocking Cx43-containing hemichannels), and the Panx1 inhibitory peptide, ^{10}Panx (100 μM), proved to be effective in this endeavor.

Given the pro-fibrotic effect of the selective $A_{2A}R$ agonist, we decided to evaluate its role on ATP release and intracellular accumulation of $[Ca^{2+}]_i$ favored by histamine (100 μM) on CGS21680 (30 nM)-treated rat subcutaneous fibroblasts cultured for 7 days. The treatment with the $A_{2A}R$ agonist increased by 9-fold the facilitatory effect of histamine

(100 μM) on ATP release from rat subcutaneous fibroblasts. The potentiating effect of CGS21680 (30 nM) on histamine-induced ATP release was prevented by the selective A_{2A} receptor antagonist, SCH442416 (10 nM). Similarly, blocking the $A_{2A}R$ favored the decline of intracellular $[Ca^{2+}]_i$ levels after the initial fast histamine (100 μM) response, an effect resembling the observed in the presence of carbenoxolone (300 μM).

In conclusion, data shows that the $A_{2A}R$ seems to exert pro-fibrotic and pro-inflammatory effects on rat subcutaneous fibroblasts, similarly to that observed in other tissues, including in human subcutaneous fibroblasts. In this context, it was demonstrated for the first time in this study that sustained activation of the $A_{2A}R$ subtype exerts a synergistic effect with histamine (and, perhaps, with other inflammatory mediators) favoring intracellular $[Ca^{2+}]_i$ accumulation and the release of ATP (danger molecule). It remains, however, to be explored the role of the adenosine $A_{2A}R$, (i) on the mechanisms regulating the release of ATP, including the expression and function of carbenoxolone-sensitive hemichannels, and (ii) on the crosstalk between subcutaneous fibroblasts, sensory nerve endings and inflammatory/immune cells invading the subcutaneous tissue under pathological contexts (e.g. dermal fibrosis, fibromyalgia and myofascial pain).

Key words: subcutaneous fibroblasts, adenosine, A_{2A} and A_3 receptors, histamine, ATP release, hemichannels, calcium mediated signaling, myofascial pain.

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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

ABC – ATP-binding cassette

AC – adenylyl cyclase

ADA – adenosine deaminase

ADO – adenosine

ADP – adenosine diphosphate

AMP – adenosine monophosphate

ATP – adenosine 5'-triphosphate

cAMP – cyclic adenosine monophosphate

CBX – carbenoxolone

CGRP – calcitonin gene-related protein

CTGF – connective tissue growth factor

Cx – connexin

DRG – dorsal root ganglia

ECM – extracelular matrix

Ecto-5'-NT – ecto-5'-nucleotidase

EGTA – ethylene-bis(oxyethylenenitrilo)tetraacetic acid

E-NTPDase/NTPDase – ecto-nucleoside triphosphate diphosphohydrolase

EPAC - exchange protein activated by cAMP

FFA – flufenamic acid

GPCR – G-protein-coupled receptor

MFQ – mefloquine

Panx – pannexin

PGP 9.5 – protein gene product 9.5

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PKB – protein kinase B

PLC – phospholipase C

SP – substance P

TGFβ-1 – transforming growth factor β-1

TH – tyrosine hydroxylase

THAPS – thapsigargin

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EUGLOH Annual Student Research Conference 2020: Rafaela Pedro Silva, Célia Soares, Carina Herman-de-Sousa, Fátima Ferreirinha, Maria Adelina Costa & Paulo Correia-de-Sá (2020): ***Unravelling Adenosine Receptors Role In Subcutaneous Fibroblasts As Novel Targets Against Myofascial Pain***. This work was presented by Universidade do Porto invitation.

Introduction

1. Nociception, a necessary evil

Nociception is an evolutionary conserved process for survival, allowing both animals and humans to sense and avoid potentially harmful stimuli. This process relies immensely on nociceptors, nowadays regarded as the first line of defense against noxious inputs (4). Nonetheless, the perception of pain was not always considered to be this complex system comprising both peripheral and central nervous systems contributions. During the 19th and early 20th century, this was a contested topic in pain sensory biology – while some were arguing that pain is a specific sensation with its own machinery, others claimed pain resulted from a central nervous system summation in response to harmful stimuli. It was not until the 1970s that the debate reached its climax, with contributions from Bessou & Perl (5) and Wall & Melzack (6) allowing to draw the conclusion that nociception is, in fact, an intricate process dependent on a vast net of mechanisms from different locations.

Nociceptors are high-threshold primary sensory neurons specialized in detecting and responding to different forms of energy in the environment, including thermic, mechanical and chemical stimuli (7). Based on their anatomical location, cell body diameter and fiber function, nociception fibers were categorized into C and A-δ fibers. Although the majority of pain afferent conducting fibers are unmyelinated and slow-conducting (C-fibers), there can also be found thinly myelinated fibers (A-δ fibers) within the periphery. Nowadays, it is widely accepted that whilst A-δ fibers mediate first, acute or sharp pain, C-fibers mediate delayed and diffuse pain (8). Regardless of their myelin constitution, conduction speed or nature of the pain mediated, all body-innervating sensory neurons have their cell bodies in dorsal root ganglia (DRG) and give rise to a single axon that bifurcates, innervating both peripheral and central nervous systems (Figure 1). The synapse from the branch innervating the central nervous system further allows the sensory stimulus to reach the brain, where it may be perceived as painful, ultimately allowing a defense response (4, 7).

Although this action-reaction process functions almost always efficiently on most people, that does not seem to be the case of chronic pain patients. In this case, the nervous system response is maladaptive (9), generating widespread pain without any noticeable input – in essence, an ongoing false alarm.

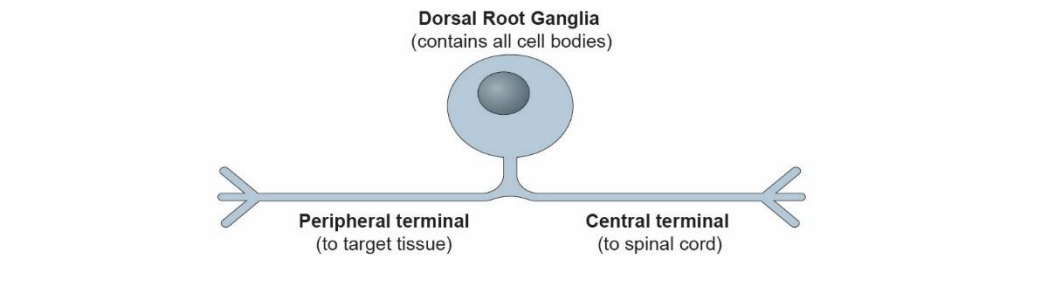


Figure 1 - Nociceptors. Schematic representation of the components of a nociceptor: a peripheral terminal innervating a particular target tissue, an axon responsible for conducting the action-potential towards the central nervous system, the DRG per se, containing all cell bodies, and a second branch, innervating the central nervous system.

a. Chronic pain syndromes

Chronic pain is a common, distressing condition that negatively impacts the life quality of its patients. Even though it usually arises from a previous injury or disease, chronic pain is considered a separate condition in its own right, easily distinguishable from acute pain (10).

As chronic pain is a wide-ranging term, the World Health Organization came further with a criterion to distinguish different types of chronic pain - from organs-related, visceral pain to muscle-, joint-related myofascial pain. Regardless of the effort to categorize different chronic pain types, pain molecular mechanisms are still unknown to date, especially in myofascial pain syndromes. These particular syndromes are characterized by the existence of sensitive points in the skin and muscles throughout the body, commonly referred to as “knots” or “trigger points”, seemingly unrelated between them. Fibromyalgia is an extreme example of such myofascial pain syndromes, where patients present more than 10 widespread trigger points and complain of disturbed sleep, stiffness, distress and fatigue (11). The muscle pain reported by fibromyalgia patients is often described as fluctuating and with tenderness, the latter most likely depending on increased peripheral and/or central nervous system responsiveness (12).

b. Peripheral and central sensitization

Increased responsiveness, sensitization or reverse tolerance, is a non-associative learning process through which an organism becomes unusually sensitive to a particular stimulus due to an amplified response (13).

In peripheral nervous system sensitization, the stimulus is mainly a set of inflammatory mediators released from injured cells, capable of reducing threshold and increasing responsiveness (4). In this scenario, a persistent and wrongful response may

emerge through two distinct routes: via the peripheral fibers or via the DRG itself (9). After being affected by metabolic damage due to cytokines or other inflammatory mediators, peripheral terminals of pain-processing, such as C and A-δ fibers, can fasten the development of pain as a result of fiber density changes and heightened nociceptor sensitivity. Further along the stimulus transduction path, axon fiber degeneration due to trauma, compression or hypoxia can result in channel expression and composition variations and, consequently, in a faulty signal transmission – DRG can also be exposed to the above listed damage, resulting in an altered membrane composition and synapse properties, culminating in a hyperexcitability of these cells (14). As a result of some of these alterations, nociceptors change from being solely responsive to noxious stimulus to also being detectors of low-intensity, innocuous input – the latter, a deep-rooted role of proprioceptive fibers such as A-α and A-β fibers (8).

After repeated or intense stimulation, the central nervous system is also susceptible to sensitization, which can occur at a spinal or a supraspinal level – involving the spinal cord or the brain, respectively. With persistent input, there seems to be a synapse enhanced efficiency due to changes in calcium permeability, receptor overexpression and synapse location at the ascending afferent neurons located in the spinal cord (15, 16); these neurons may even release inflammatory intermediaries, activating microglia cells which in its turn release pain-promoting mediators (e.g., chemokines, growth factor, proteases) (17). At supraspinal regions, however, the main pathway of sensitization occurs through a misbalance between descending facilitation and inhibition, in which facilitation prevails (9).

Due to their polymodal nature, C fibers are more prone to suffer peripheral sensitization, as its activation by chemical stimuli such as histamine and H^+ facilitates the production of substance P (SP) and calcitonin gene-related protein (CGRP), pro-inflammatory mediators (8). As a direct consequence, these fibers are responsible for mediating delayed, diffuse pain, which may endure long after the inflammation process has ceased. Recent findings from Coppi *et al* suggest the presence of nucleoside-selective purinergic receptors in DRG neurons (18), opening a new chapter on chronic pain syndromes drug development and treatment.

2. Fascia, a nonspecialized connective tissue, and its implications in pain perception

Upper mammalian animal bodies are predominantly formed by four types of biological tissues, namely epithelial, muscular, nervous and connective tissues. Particularly, the connective tissue is composed of specialized and non-specialized cells, both including specific cells and extracellular matrix (ECM) as main components. Specialized connective tissues comprise the adipose tissue, cartilage, bone and blood, whereas non-specialized tissues, also referred to as fascia, are regarded as undifferentiated mesenchymal tissues that surround other body structures (19, 20). Fibroblasts are the most predominant cells in non-specialized tissues, being responsible for keeping the ECM homeostasis (21, 22).

Due to its lack of compartmentalization, fascia is difficult to define. According to its Latin origin, fascia means “bundle, unification or binding together” (23), but traditionally, it has been defined as the “term applied to masses of connective tissue large enough to be visible to the unaided eye” (24). Since 2007, fascia has been described as “the soft tissue component of the connective tissue system that permeates the body” (25), but consensus on this designation is yet to be accomplished by the medical community. This soft tissue is part of all organ systems, including the cardiovascular, gastrointestinal, neurological and musculoskeletal systems, to name a few. Since it is so widespread among different systems, there is no surprise that several fascial structures exist, each one with different densities and proportions of its main component – collagen.

a. Fascia as a sensory organ

So far, not much is known about the existence of sensory nerve fibers in non-specialized connective tissue. One of the first discoveries in this field was accomplished by Stilwell (26), and later Hirsch (27), whom identified large Pacinian corpuscles, one of the main four types of mechanoreceptors, and free nerve endings in human fascia. Later, Yahia further demonstrated the existence of another type of mechanoreceptor in human lumbar fascia, namely Ruffini corpuscles (28). Despite this interesting data, it was still difficult to conclude whether the fascia was itself innervated or whether the nerve fibers simply lied on fascia in association with it (20).

In more recent years, a clear innervation of fascia has been demonstrated. Rat fascia appears to be highly innervated by unmyelinated and myelinated afferents with different

diameters and velocities, found to be within the range of C, A-δ and A-β fibers (29), and proven to be sensitive to mechanical and chemical noxious stimulation (30, 31). Moreover, some of these nerve fibers were demonstrated to be immunoreactive against the neuronal marker protein gene product 9.5 (PGP 9.5), tyrosine hydroxylase (TH), CGRP and SP (29) – the latter two being involved in nociception pathways (8, 32). While evaluating sensory nerve fibers terminating within fascia, Corey and coworkers were the first to identify "sensory fibers terminations within the collagen matrix of connective tissue in the low back". These fibers, which expressed positive CGRP immunoreactivity, were then proven to be DRG cells (33). Altogether, these findings suggest there is sensory innervation in non-specialized connective tissue, including mechanosensory and nociceptive neurons, which may be partly responsible for one's perception of pain.

b. Role in musculoskeletal pain

The wide spread nature of fascia throughout the body and its plasticity, alongside its mechanical role and the ability of fibroblasts to communicate with each other via gap junctions, suggests fascia is most likely to act as a body-wide mechanosensitive signaling system, with an integration function equivalent to that of the nervous system (34). This idea has been further supported by recent evidence in which it was found that during pathophysiological states, for instance, myofascial pain syndromes, the subcutaneous tissue is altered. In such conditions, tissue remodeling appears to lead to an increased thickness and fibrosis, that is, a pathological accumulation of ECM proteins (35). Despite recent developments, the mechanism by which this alteration occurs is not yet fully understood.

In myofascial pain syndromes, the connective tissue that surrounds and infiltrates the muscles might be remodeled in response to mechanical stress, including the lack of movement or, contrariwise, its overuse (35). While the absence of movement promotes tissue atrophy and disorganization, its overuse usually leads to injury and inflammation; nevertheless, both types of mechanical stress inevitably end up with tissue fibrosis. Regarding overuse, for example when muscles are under constant movement, fascia is submitted to microinjuries. Consequently, subcutaneous fibroblasts secrete IL-6 (36) and promote tissue inflammation, which includes transforming growth factor β-1 (TGFβ-1) production, an anti-inflammatory cytokine and powerful inducer of collagen synthesis; during this process, a few cells may undergo apoptosis. As a direct result of cell death, several molecules are released into the extracellular space, namely ions (H^+, K^+),

adrenaline, SP, CGRP, bradykinin and histamine – all known components of the so called “inflammatory soup” (8). Histamine release further stimulates TGFβ-1 production, culminating in tissue fibrosis (Figure 2) (35).

Patients with myofascial pain syndromes have been shown to have hyperalgesia, suggesting abnormal central pain processing (35, 37). However, this may be due to nociceptive neurons constant activation and peripheral sensitization, which then contributes to the development or worsening of the inflammation and, consequently, fibrosis (35). Being aware of this, one should ask how can a nociceptor be peripherally sensitized. Until now, it remains unclear whether myofascial “trigger points” are the cause or the effect of injury and pain. Since these “knots” represent contracted muscle fibers, it is possible that signaling molecules essential to the onset of inflammation wrongfully accumulate and activate nearby nociceptors. In this scenario, myofascial pain syndromes would arise from a miscommunication between subcutaneous fibroblasts and sensory afferent fibers. Hence, fascia may hold one of the keys for understanding and treating musculoskeletal pain.

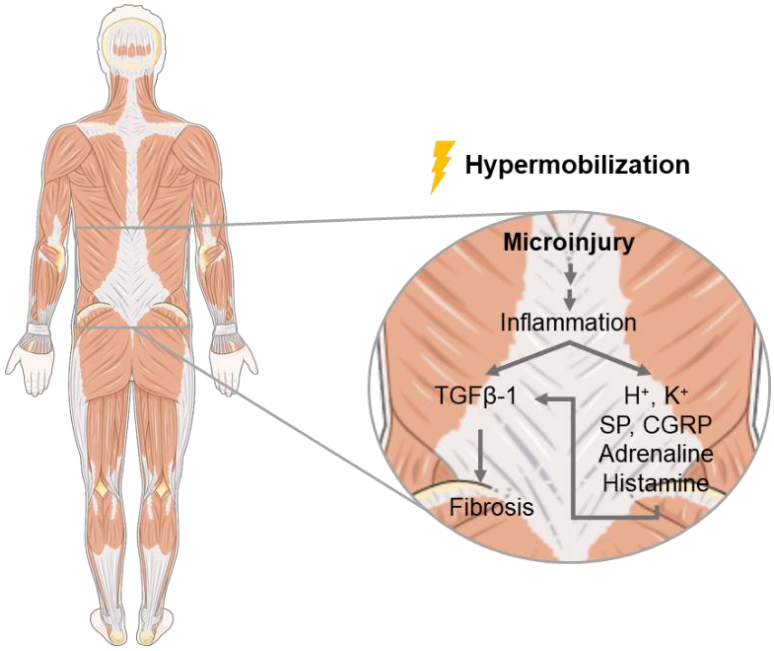


Figure 2 – Pathophysiological model for myofascial pain syndromes.

3. Purines as versatile molecules and key life factors

Purines, a broader class of purine-derived molecules, are water-soluble ubiquitous substances found in both prokaryotic and eukaryotic cells. Consisting of a heterocyclic aromatic compound with joined pyrimidine and imidazole rings, purines are the most commonly nitrogen-containing heterocycles found in nature. Apart from their role in DNA and RNA, purines are important in other biomolecules' synthesis, such as ATP and cyclic AMP (cAMP).

Currently, it is well accepted that ATP can act as a signal molecule that affects cell behavior besides its major role in providing energy for the cell. Yet, the "purinergic signaling dynamics" hypothesis, where Geoffrey Burnstock proposed ATP as a neurotransmitter (38), firstly met substantial resistance in the 1970s. This opposition was mainly due to ATP being established as an intracellular-contained molecule whose sole purpose was to provide energy for the cell to function. Later on, studies regarding this possible new role of ATP increased, demonstrating its importance not only in phenomena such as glial growth, proliferation and survival as an intracellular messenger (39), but also in communication between nerve cells and muscle fibers as an extracellular messenger (40). Nowadays, ATP is well-established as a neurotransmitter, neuromodulator and co-modulator (41), present in both intra- and extracellular compartments even if to a different extent. While in the intracellular compartment ATP concentration is within the mM range, extracellular concentrations of this nucleotide are maintained low at physiological conditions, specifically within the nM to μM range (42).

a. Nucleotide- and nucleoside-releasing pathways

Cellular release of endogenous nucleotides depicts a major component of the purinergic signaling cascade. For many years, cell lysis due to damaged or dying cells was thought to be the main mechanism through which cells released massive amounts of nucleotides. However, we now know this is a nonspecific mechanism, being restricted to injury, shock and inflammatory conditions (42, 43).

The diversity of conditions in which cells release ATP or adenosine diphosphate (ADP) implicates multiple pathways. These cellular mechanisms include (1) electro diffusional movement through plasma membrane ion channels, including Cx and Panx hemichannels, (2) facilitated diffusion by nucleotide specific ATP-binding cassette (ABC) transporters, and (3) cargo-vesicle trafficking and exocytotic granule secretion (42, 44, 45) (Figure 3). Besides their major role in nucleotide extrusion, Cxs are also implicated

in intercellular communication through gap junctions, an essential process to coordinate survival or death cellular responses not only on physiological, but also on pathological scenarios (46).

A non-lytic method, namely vesicular exocytosis, often depends on Ca^{2+} availability in a wide range of both excitable (i.e., nerve cells) and non-excitable cells (e.g. fibroblasts (40), epithelial cells, neutrophils, monocytes and macrophages) – the latter being transiently promoted by stimuli such as shear stress, hypoxia and pharmacological agents, like bradykinin and histamine.

Regarding smaller nucleotides, such as AMP, it remains uncertain whether this nucleotide is directly released from stimulated-cells or if it accumulates as an intermediate metabolite from more complex nucleotides (42).

Nucleosides such as ADO, however, can be found in the extracellular milieu either via direct release by equilibrative nucleoside transporters or as a consequence of extracellular ATP/ADP enzymatic breakdown. While stress, hypoxia and inflammation increase extracellular adenosine levels up to micromolar concentrations, the catabolism of adenine nucleotides is the main ADO source in the extracellular milieu (42).

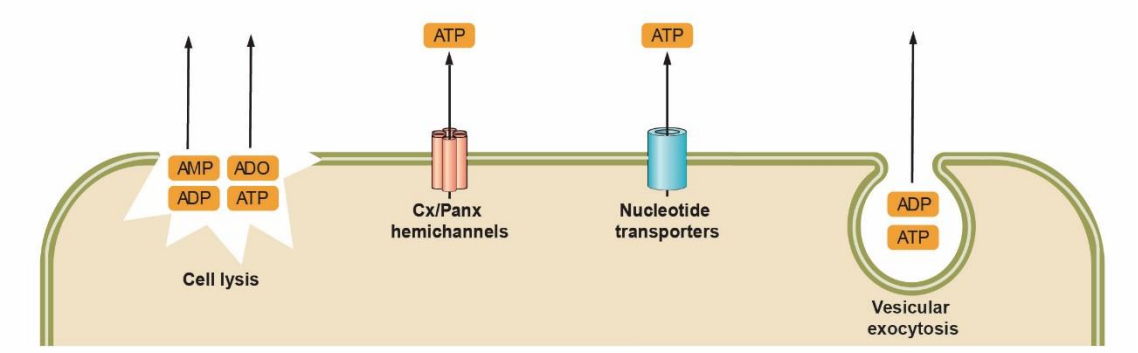


Figure 3 – Nucleotide- and nucleoside- releasing pathways. Besides heavy nucleotide leakage upon cell lysis or damage, nucleotides can occur at the extracellular milieu via Cx/Panx hemichannels, nucleotide transporters and vesicular exocytosis.

b. Extracellular nucleotide catabolism as an adenosine producer

Purinergic signaling appears to be far more complex than any other neurotransmitter, as its signal transduction mechanisms are strictly dependent on the levels of its messengers, which are, ultimately, modulated by the activity of colocalized nucleotide-hydrolytic enzymes. ATP is not transported out of the synaptic cleft or extracellular space, but it is instead hydrolyzed, producing other purine-derived molecules such as

ADP, AMP and ADO. Consequently, ATP exerts a dual function, being able to directly activate ATP-sensitive P2X and/or P2Y receptors and, after its breakdown into ADP and ADO, further activate other P2Y as well as adenosine-sensitive P1 receptors, respectively. General schemes for nucleotide hydrolysis and response termination include a role for up to 4 enzymes, being the most relevant to this project E-NTPDase/NTPDase and Ecto-5'-NT.

Once in the extracellular milieu, nucleotide breakdown to ADO is achieved by two specific and consecutive ecto-nucleotidase reactions, in which E-NTPDase and ecto-5'-NT enzymes are equally important. E-NTPDases 1, 2, 3 and 8 are large membrane-bound enzymes with their catalytic side facing the extracellular milieu, which are responsible for hydrolyzing 5'-tri or diphosphates to their respective 5'-di- and monophosphate nucleosides. Adenine nucleotides are therefore hydrolyzed as the following:

$$ATP \rightarrow ADP + P_i \qquad \text{and/or} \qquad ADP \rightarrow AMP + P_i$$

For optimal catalytic activity, millimolar concentrations of Ca^{2+} and Mg^{2+} are required (47). The second and final step in this chain-reaction of nucleotide hydrolysis is accomplished by Ecto-5'-NT. While the majority of enzymes belonging to this family can be found within the cell and have a low affinity for its substrate, there is a surface-associated enzyme, else known as CD73 (48), capable of promptly hydrolyzing AMP:

$$AMP \rightarrow ADO + P_i$$

4. Adenosine-sensitive P1 purinoceptors

Only a few years later after Burnstock coined the "purinergic signaling" hypothesis, a two-subfamily division of the receptors based on their different ligand binding was proposed and accepted. From that moment on, purinergic effects were known to be mediated via a series of nucleoside- (ADO) and nucleotide- (ATP, ADP, uracil-containing nucleotides and some other dinucleotides) sensitive receptors, designated as P1 and P2 purinoceptors, respectively. Regarding the subject of the present thesis, only P1 receptors will be further addressed.

Adenosine-sensitive P1 purinoceptors are metabotropic receptors coupled to several proteins of the heterotrimeric guanine nucleotide-binding family (GPCR), being divided

into four already cloned subtypes - A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R - according to their distinct functional and pharmacological characteristics.

All receptors are composed of 7 transmembrane α-helices, followed by an extracellular N-terminus and a cytosolic C-terminus, as well as 3 extracellular loops and 3 intracellular loops. Although the overall sequence similarity is somewhat high among these, each receptor subtype has its unique ligand binding profile, subcellular location and G-protein binding preference, consequently suggesting different physiological implications (49-51).

$A_{2A}R$ and $A_{2B}R$ are both positively coupled to adenylyl cyclase (AC), with the former exhibiting much higher affinity for ADO (0,1 – 1,0 μM) than the latter (52). A_1R is negatively coupled to AC and is able to modulate K^+ channels activity; in some cases, this receptor can even positively modulate phospholipase C (PLC). While A_3R receptor is able to diminish cAMP cell levels, this receptor can also positively couple to PLC (51).

a. Adenosine receptors role in pain and nociception

Over the past two decades, ADO has emerged as a dual molecule, capable of both relieving and producing pain, depending on receptor specific-tissue expression and activity (53). In this context, ADO or ADO receptor agonists are not presently being used to treat chronic pain conditions, although they are being clinically studied to achieve this goal.

First insights regarding the role of ADO in pain conditions focused on the activity of the A_1R, the smallest among P1 purinoceptors, given its abundance and widespread distribution among pain-perceiving neuronal cells at the dorsal horn of the spinal cord (54). When injected intrathecally in rodent models, it mediates antihyperalgesic and antiallodynic effects - hyperalgesia being increased sensitivity to pain, and allodynia a condition where pain is caused by no apparent stimulus (55) - even though some side effects such as headaches and low back pain were reported. In light of this evidence, efforts were made to develop a systemic method of drug delivery. Unfortunately, oral or intravenous A_1R agonists delivery methods failed, as this receptor is highly expressed in the atrioventricular and sinoatrial nodes of the heart and its activation leads to slower, sometimes critical, heart rates (53). In 2010, the A_1R popped-up again given its antinociceptive effects on acupuncture (56), yet little attention has been paid since then.

Failing to prove A_1R worth in treating chronic pain, researchers next turned to the low affinity $A_{2B}R$. Since this particular receptor has low expression levels in the central

nervous system while it is abundant in immune and inflammatory cells (57), there was not much hope about this receptor being involved in nociception. Despite late $A_{2B}R$ characterization, low affinity to ADO and lack of highly selective agonists have hampered studies regarding its role in pain perception. Notwithstanding few preliminary findings showing a possible antinociceptive effect when resorting to receptor antagonists (58), the $A_{2B}R$ has been discarded mainly due to its promiscuity.

Later on, the A_3R emerged as a possible pain-promoting receptor, regardless of its low central nervous system expression (57). Early studies on A_3R deficient rodents emphasize that these animals showed less hyperalgesia and inflammation markers, being consistent with a pronociceptive role of this receptor (59). However, administration of a selective A_3R agonist produced a spinal antinociceptive effect (60), leaving scientists puzzled. The role of this particular receptor remained mysterious for several years, when in 2019 it was reported that the A_3R activation leads to a pronociceptive effect inhibition on neuronal cells (18).

The $A_{2A}R$ is perhaps the most extensively studied ADO receptor until today. It is the biggest receptor of the P1 purinoceptor family comprising as much as 412 amino acids; the $A_{2A}R$ shares about 80% of sequence homology between the human and mouse or rat species (61). This receptor distribution to the plasma membrane appears to be depended on the glycosylation of a specific residue, and receptor regulation depends on Thr298 phosphorylation by receptor kinases (49, 61). Signaling through the $A_{2A}R$ relies on its coupling to G_s and stimulation of AC, which then activates downstream effectors, such as protein kinase A (PKA) and cyclic nucleotide-gated ion channels (49, 50, 62). Other effects, including mobilization of intracellular calcium, have also been reported (61), but the majority of downstream events are yet to be fully understood. Regarding function, this receptor subtype is classically known to modulate postsynaptic neuronal activity by regulating neurotransmitter release, especially in GABAergic neurons, throughout both central and peripheral nervous systems. Apart from being selectively expressed in certain brain regions, it is also expressed on glial cells, having showed a potential beneficial or protective effect on several central nervous system disorders, namely Parkinson's (63) and Alzheimer's (64) diseases, when resorting to receptor antagonists. Furthermore, the $A_{2A}R$ is also found in blood platelets and in most lymphoid cells (e.g., lymphocytes and granulocytes), being considered a target for inflammatory and immune conditions (65). Pertaining to pain, the role of this particular receptor has been ambiguous, as studies have shown both pro- and antinociceptive roles. For instance, the presence of the $A_{2A}R$ in both DRG neurons and peripheral endings,

supports a pronociceptive role (66, 67); yet, $A_{2A}R$ antagonist administration abolished antinociceptive effects of intrathecally administrated ADO in rats (60).

b. Adenosine receptors role in tissue healing and fibrosis

Tissue disorganization is a frequent element in patients with chronic pain, feasibly as a result of tissue inflammation and fibrosis (35, 68, 69). Chronic deformation of the myofascial system, namely increased deposition of collagen, causes mechanoreceptors to convert into nociceptors, ultimately allowing the development of chronic pain. Fibroblasts, the major component of the myofascial system (21, 22), play a key role in transmitting nociceptive information, crucial for the appropriate functioning of a given organism.

Evidence shows that activation of the same receptor subtype may cause opposing effects depending on the tissue. For example, the adenosine $A_{2B}R$ promotes fibrosis in the lung whilst inhibiting fibrosis in the heart (70). Furthermore, both $A_{2A}R$ and $A_{2B}R$ appear to promote fibrosis in the skin, liver and lungs (as reviewed by (71)). Nonetheless, ADO receptors expression and activation in pathophysiological states such as inflammatory conditions and tissue remodeling/fibrosis are still dubious (72).

5. Previous Findings

Previous findings from our group demonstrated that human subcutaneous fibroblasts release ATP in response to mechanical or noxious chemical stimuli, such as bradykinin (1) and histamine (2). Once at the extracellular milieu, ATP is promptly converted to AMP by NTPDase1/2 and, subsequently, into ADO by Ecto-5'-NT at the surface of human subcutaneous fibroblasts. Moreover, ADA shows low extracellular activity under stressful conditions resulting in extracellular ADO accumulation and P1 receptors activation. Among these, the $A_{2A}R$ and A_3R subtypes are the most expressed P1 receptors in human subcutaneous fibroblasts (3).

Bearing in mind that controversy still exists regarding the role of ADO in subcutaneous tissue healing and remodeling, as well as its implication in myofascial pain, we decided to investigate the effects of P1 receptor subtypes on rat subcutaneous fibroblasts under physiological and histamine-induced inflammatory conditions, given to the fact that the nucleoside is generated and accumulates in the subcutaneous tissue milieu under stressful conditions.

Objectives

The main goal of this study was to unravel the ADO role on growth and differentiation of rat subcutaneous fibroblasts behavior under both physiological and histamine-induced inflammatory conditions. The ultimate goal (yet to be undertaken) is to understand how these cells might communicate with sensory nerve fibers involved in nociception triggered by disturbances of the subcutaneous connective tissue.

Having this in mind, the following objectives were undertaken:

I. Assess the differential expression of P1 receptor subtypes in rat subcutaneous fibroblasts;
II. Characterize the effects in growth and differentiation of the most expressed P1 receptor subtypes, $A_{2A}R$ and A_3R, on rat subcutaneous fibroblasts primary cultures;
III. Evaluate whether activation of P1 receptor subtypes, namely the $A_{2A}R$ receptor, on histamine-induced intracellular Ca^{2+} oscillations and ATP release, as readouts of the ADO influence on the inflammatory response operated in subcutaneous fibroblasts.

Materials and Methods

1. Drugs and Solutions

2-p-(2-Carboxyethyl) phenethylamino-5′-N-ethyl carboxamido adenosine hydrochloride hydrate (CGS21680), Direct Red 80, ethylene-bis(oxyethylenenitrilo)tetra acetic acid (EGTA), N-(α,α,α-Trifluoro-m-tolyl)anthranilic acid, N-(3[Trifluoromethyl] phenyl) anthranilic acid (FFA), 2-(4-imidazolyl)ethylamine (HIST), 2-Piperidinyl-2,8-bis(trifluoromethyl)-4-quinolinemethanol monohydrochloride (MFQ), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT), phosphate-buffered saline solution (PBS), trypsin-EDTA, 2,4,6-Trinitrophenol (picric acid) and other cell culture reagents such as amphotericin B, DMEM-high glucose medium (DMEM), fetal bovine serum (FBS), gentamicin, penicillin/streptomycin and were obtained from Sigma-Aldrich (St. Louis, MO, USA). (3β,20β)-3-(3-Carboxy-1-oxopropoxy)-11-oxoolean-12-en-29-oic acid disodium (CBX), ^{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), 6-(Acetyloxy)-2,3,3a,4,5, 6,6a,7,8,9bdecahydro-3,3a-dihydroxy-3,6,9-trimethyl-8-[[(2*Z*)-2-methyl-1-oxo-2-butenyl]oxy]-2-oxo-4-oxobutoxy)azuleno[4,5*b*]furan-7-yl octanoate (Thapsigargin) were purchased from Tocris BioScience (Bristol, UK). Bouin liquor was supplied by PanReac AppliChem ITW Reagents (Barcelona, Spain). Ionomycin was acquired from AbCam (Cambridge, UK) and Fluo-4NW kit was purchased from Molecular Probes (Invitrogen, California, USA). ATP bioluminescence assay kit HS II was acquired from Roche Applied Science (Penzberg, Germany).

2. Animals

Subcutaneous fibroblasts were obtained from male Wistar Han rats (3-6 months old, Charles River™, Barcelona, Spain). Animal care and experimental procedures were performed in consonance with the United Kingdom Animals (Scientific Procedures) Act 1986 and followed the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for Care and Use of Laboratory animals (NIH Publications No. 80-23) revised 1996. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. The study was approved by the Ethics Committee and the Animal Welfare Responsible Organism of ICBAS-UP (Decision no 224/2017). Wistar rats were kept at a constant temperature (21°C) and a regular light (06.30–19.30 h)–dark (19.30–06.30 h)

cycle, with food and water *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3. Cell cultures

Fibroblasts were isolated from the subcutaneous tissue of Wistar rats by the explant technique. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2.5 μg/mL amphotericin B, 100 U/mL of penicillin/streptomycin and 50 μg/mL gentamicin; cell cultures were kept at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Media were replaced twice a week. Primary cultures were maintained until near confluence (3 to 4 weeks), after which adherent cells were enzymatically released with 0.04% trypsin-EDTA solution. The resultant suspension was seeded (first subculture) and maintained in the same conditions as mentioned above. All experiments were performed using the first subculture.

4. Immunocytochemistry

Rat subcutaneous fibroblasts were seeded in chamber slides at a density of $3x10^4$ cells/mL and allowed to grow for 7 days. Cultured cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, after which they were washed 3 times in PBS for 10 minutes and incubated with blocking buffer I (10% FBS, 1% bovine serum albumin (BSA), 0,1% Triton-X and 0.05% NaN_3) for 1h. Primary antibodies, diluted in blocking buffer II (5% FBS, 1% BSA, 0,1% Triton-X and 0.05% NaN_3), were added and incubated overnight at 4ºC [rabbit anti-rat A_1 (1:50, Chemicon, CA, USA), rabbit anti-rat A_{2A} (1:100, Alpha Diagnostic, TX, USA), rabbit anti-rat A_{2B} (1:50, Alomone, Jerusalem, Israel), rabbit anti-rat A_3 (1:50, Alomone, Jerusalem, Israel)]. Cells were then washed 3 times in PBS for 10 minutes. The anti-rabbit Alexa 488 secondary antibody (Molecular Probes, OR, USA) was diluted (1:1000) in blocking buffer II and applied for 1h at room temperature, protected from light. One last wash using PBS was performed, after which glass slides were mounted using VectaShield medium and stored at 4ºC. Images were then acquired in an Olympus FV1000 confocal microscope (Tokyo, Japan) at a 40x magnification.

5. Cell viability and proliferation

Viability and proliferation were assessed by the MTT assay (1, 2). This assay consists on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrasodium bromide (MTT) to a purple formazan reaction product by viable cells. Rat fibroblasts were seeded in 96-

well plates at a density of $3x10^4$ cells/mL and cultured in supplemented DMEM medium, as described above. Cell cultures were regularly monitored and characterized at days 1, 7, 14 and 21 of the first subculture. Cells were incubated with 0,5 mg/mL of MTT for 4h in a humidified chamber, after which the medium was carefully removed, and the stained product was dissolved using dimethyl sulfoxide (DMSO). Absorbance determination was performed at 600 nm using a microplate reader spectrometer (Synergy HT, BioTek Instruments).

6. Total collagen determination

Total collagen determination was performed using the Sirius Red assay. Rat fibroblasts were cultured and monitored as described for the viability/proliferation assay. This staining assay was performed using minor alterations to the protocol established by Reinert *et al* (73). Cell layers were washed twice with PBS before fixation with Bouin for 1h. The fixation fluid was later removed, and the culture plates were washed by immersion in running tap water for 15 minutes. Plates were then allowed to air dry before adding the Sirius Red dye (Direct Red 80), after which the cells were stained for 2h under mild shaking. To remove the non-bound dye, stained cells were washed with 0,01 M hydrochloric acid, followed by distilled water. The bound dye was later dissolved in 0,1 M sodium hydroxide and absorbance was measured at 550 nm using a microplate reader spectrometer (Synergy HT, BioTek Instruments).

7. Extracellular ATP quantification by bioluminescence

Extracellular ATP was quantified by the luciferin-luciferase ATP bioluminescence assay kit HS II (Roche Applied Science), using a multi detection microplate reader (Synergy HT, BioTek Instruments). Cells were seeded in a flat bottom 96 well plates at a density of $3x10^4$ cells/mL and allowed to grow for 7 days. At the beginning of each experiment, cells were washed twice with gassed (95% O_2 and 5% CO_2) Tyrode's solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 11.2, at 37ºC, before collecting the first sample (T_1, time zero). The cells were, then, allowed to equilibrate with Tyrode's solution for 30 minutes, after which the second sample was collected (T_2 basal, time 30 min). This procedure was repeated in the presence of histamine (100 μM); after a 30-min incubation with histamine a third sample was collected (T_3 histamine, time 60 min). Collected samples were immediately stored at -80ºC until ATP measurements.

For ATP determinations, the samples were defrosted till 25ºC and then, mixed with the luciferin-luciferase ATP bioluminescence assay kit according to manufacturer instructions. Bioluminescence of the samples was compared with that obtained using high-purity external ATP standards within the 0.1 nM – 1 mM range; all measurements were done in duplicate. Bioluminescence assays were done at room temperature; light emission acquisition was performed 20 seconds after addition of the luciferin-luciferase mixture to the collected sample.

8. Measurement of intracellular $[Ca^{2+}]_i$

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured with the calcium sensitive dye Fluo-4 NW (Fluo-4 NW calcium assay kit, Molecular Probes, Invitrogen) in a multi detection microplate reader (Synergy™ HT Multi-Mode Microplate Reader, BioTek Instruments) (74). Rat subcutaneous fibroblasts were seeded in flat bottom 96 well plates at a density of $3x10^4$ cells/mL. Cells were cultured for 7, 21 or 28 days (as required) in supplemented DMEM medium, as previously described. On the day of the experiment, cells were washed twice with gassed (95% O_2 and 5% CO_2) Tyrode's solution containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 11.2 (pH 7.40), at 37ºC for 30 minutes, with the cell-permanent calcium fluorescent indicator Fluo-4NW (2,5 μM). After removing the fluorophore solution, cells were once again washed twice with the Tyrode's solution, after which 150 μL of this same solution was added per well. For the recordings, temperature was set to and maintained at 32ºC and readings were obtained every 5 seconds, during roughly 30 minutes. Using a tungsten halogen lamp, fluorescence was excited at 485/20 nm and emission was measured at 528/20 nm. All calcium measurements were calibrated to the maximum calcium response produced by the Ca^{2+} ionophore, ionomycin (5 μM, 100% response) (75).

9. Presentation of data and statistical analysis

Data are hereby shown as mean ± SEM (Standard Error of Mean), with n representing the number of animals used in each procedure. Statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, California, USA). Statistical significance was evaluated with student's t-test and one-way ANOVA, with statistically significant values defined by a p-value of less than 0.05.

Results

1. Characterization of rat fibroblast cells in culture

Figure 4A shows that rat fibroblast cultures grown for 21 days in control conditions exhibited a gradual rise in cell viability/proliferation (MTT assay) throughout the test period. Results concerning collagen production (Sirius Red assay) (Figure 4B) followed a similar pattern to that obtained in the MTT assay, indicating that under the present experimental conditions, the amount of extracellular matrix being produced depends directly on the number of viable cells in the culture.

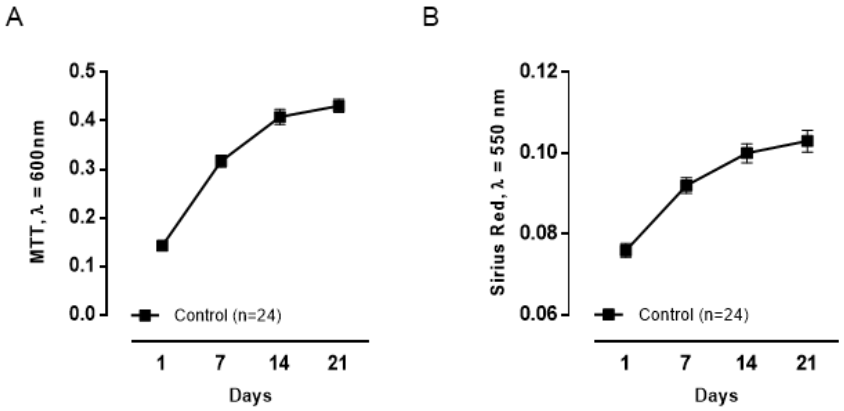


Figure 4 - Viability/proliferation (A) and collagen production (B) by cultured fibroblasts of the rat subcutaneous tissue. Isolated cells were grown in culture for 21 days. Each point represents pooled data from 24 individuals; 3-4 replicas were performed for each individual. Vertical bars represent SEM and are shown when they exceed the symbols in size.

2. P1 receptors expression in rat subcutaneous tissue

Rat subcutaneous fibroblasts are elongated cells with a characteristic spindle-shaped morphology (Figure 5). From the four adenosine receptor subtypes, rat subcutaneous fibroblasts grown in culture for 7 days show strong immunoreactivity against $A_{2A}R$ and A_3R subtypes, with no evidence of the A_1R being present in these cells (Figure 5). Regarding the $A_{2B}R$ immunoreactivity, it shows a faint staining in the cytoplasm and strong reactivity in the nucleus; functional meaning of this receptor localization remains to be explored in future studies. The low immunoreactivity against A_1R and $A_{2B}R$ 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 (76).

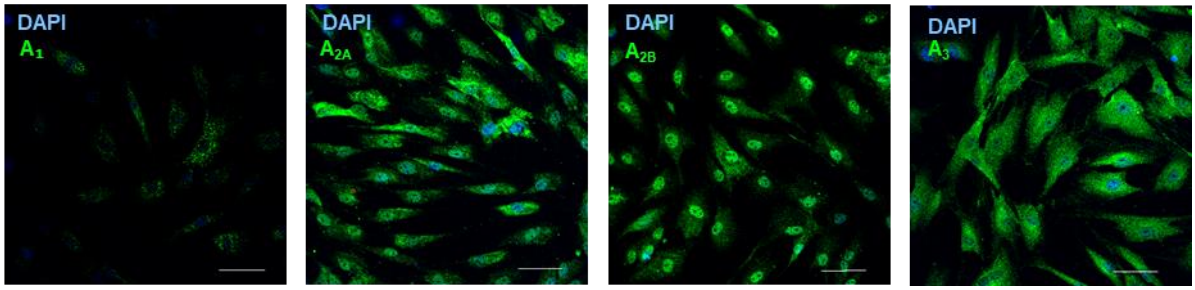


Figure 5 - Rat subcutaneous fibroblasts express high amounts of adenosine A_{2A}R and A_3R, with very little amounts of A_1R and A_{2B}R subtypes. Shown are immunofluorescence confocal micrographs using specific antibodies against A_1R, A_{2A}R, A_{2B}R and A_3R receptor subtypes. Magnification: 40x; Scale bar is 50 μM.

3. Effects of A_{2A} and A_3 receptors activation on growth and total collagen production by rat subcutaneous fibroblasts

Considering that A_{2A}R and A_3R were the most represented P1 receptors subtypes in rat subcutaneous fibroblasts, we set out to evaluate their role on growth and total collagen production by these cells.

Incubation of rat subcutaneous fibroblasts with the A_3R agonist, 2-Cl-IB-MECA (0.01-1 μM), for 21 days concentration-dependently increased ($p<0.05$) cells growth/viability (MTT assay; Figure 6A), without much affecting the collagen production (Sirius Red assay; Figure 6B). The proliferating effect of 2-Cl-IB-MECA (1 μM) at day 21 was prevented by blockage of the A_3R with MRS1191 (10 nM) (Figure 7).

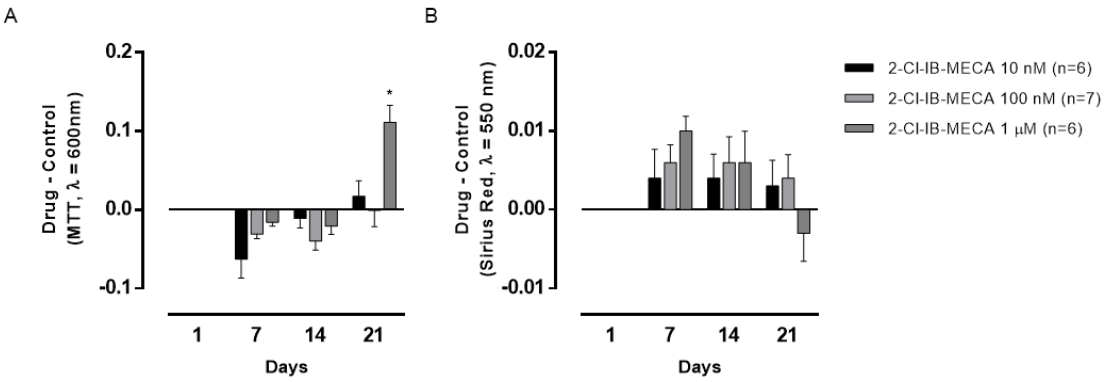


Figure 6 - Effects of the adenosine A_3R agonist, 2-Cl-IB-MECA (0.01-1 μM), on proliferation/viability (MTT assay, A) and collagen production (Sirius Red staining, B) by rat subcutaneous fibroblasts grown in culture for 21 days. Each bar represents pooled data from 6-7 animals; 3-4 replicas were performed for each individual. Vertical bars represent SEM. * $p<0.05$ (one-way ANOVA) represent significant differences compared to control conditions.

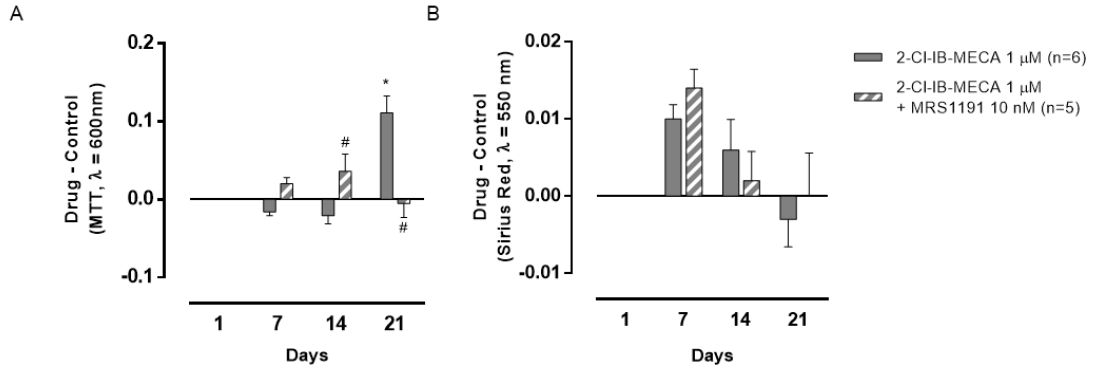


Figure 7 - Selective blockage of adenosine A_3R with MRS1191 (10 nM) prevents 2-Cl-IB-MECA (1 μM)-induced proliferation of rat subcutaneous fibroblasts grown in culture for 21 days. Each bar represents pooled data from 5-7 animals; 3-4 replicas were performed for each individual. Vertical bars represent SEM. * p<0.05 (one-way ANOVA) represents significant differences compared to control conditions and # p<0.05 (t-test) depicts differences compared to the effect of 2-Cl-IB-MECA alone in the same experiments.

The selective A_{2A}R agonist, CGS21680 (10-100 nM), concentration-dependently increased (p<0.05) cells growth/viability (MTT assay; Figure 8A) by rat subcutaneous fibroblasts at culture day 21. A similar tendency was also observed regarding collagen production (Sirius Red assay; Figure 8B). The pro-fibrotic effect of CGS21680 (30 nM) was prevented by co-application of the selective A_{2A}R antagonist, SCH442416 (10 nM) (Figure 9).

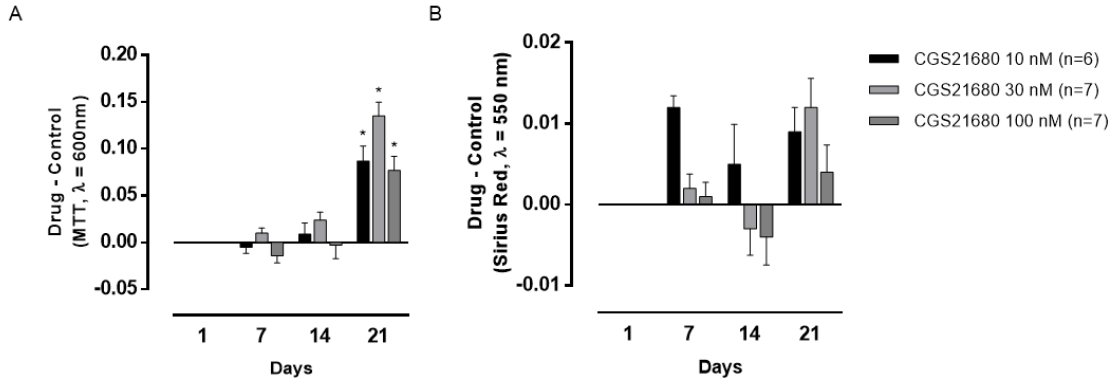


Figure 8 - Effects of the adenosine A_{2A}R agonist, CGS21680 (10-100 nM), on proliferation/viability (MTT assay, A) and collagen production (Sirius Red staining, B) by rat subcutaneous fibroblasts grown in culture for 21 days. Each bar represents pooled data from 6-7 animals; 3-4 replicas were performed for each individual. Vertical bars represent SEM. * p<0.05 (one-way ANOVA) represents significant differences compared to control conditions.

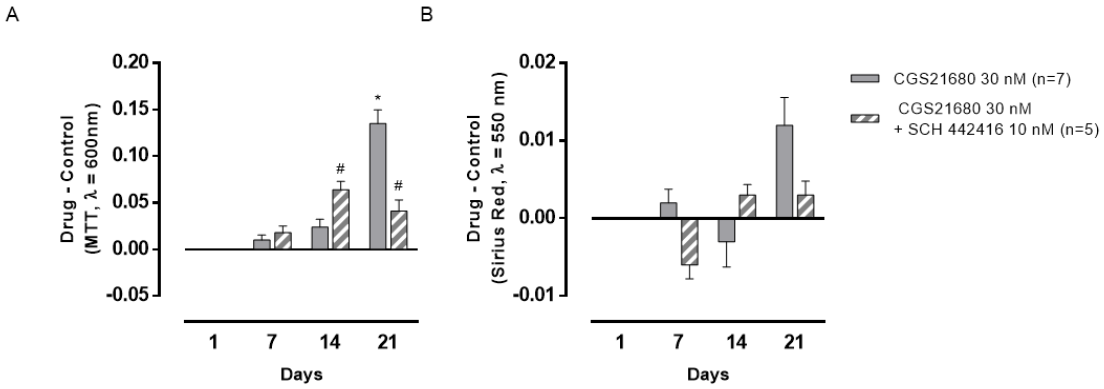


Figure 9 - Selective blockage of adenosine A_{2A}R with SCH442416 (10 nM) prevents CGS21680 (30 nM)-induced proliferation of rat subcutaneous fibroblasts grown in culture for 21 days. Each bar represents pooled data from 5-7 animals; 3-4 replicas were performed for each individual. Vertical bars represent SEM. * p<0.05 (one-way ANOVA) represents significant differences compared to control conditions and # p<0.05 (t-test) depicts differences compared to the effect of CGS21680 alone in the same experiments.

4. Histamine favors cells growth, intracellular Ca^{2+} accumulation and ATP release by rat subcutaneous fibroblasts

Previous reports from the literature demonstrate that the inflammatory mediator histamine promotes fibrosis in a number of different tissues (e.g. lung and skin) (77, 78). Our group provided compelling information that it also favors growth, intracellular $[Ca^{2+}]_i$ accumulation and ATP release by human subcutaneous fibroblasts (2). Figure 10 shows that histamine (100 μM) increased growth (p>0.05) (and collagen production) by rat subcutaneous fibroblasts at culture day 21.

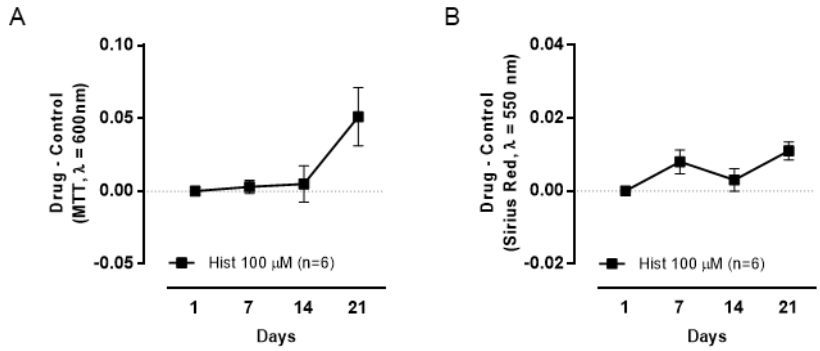
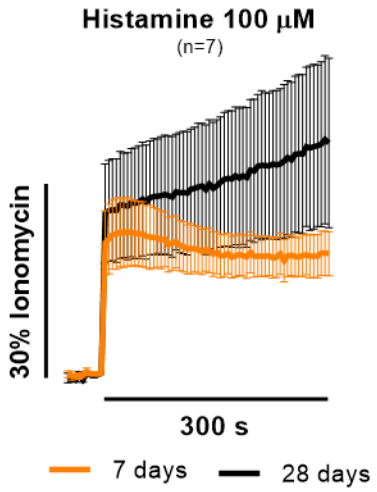


Figure 10 – Effect of histamine (100 μM) on cells growth (MTT assay, A) and collagen production (Sirius Red staining, B) by fibroblasts of the rat subcutaneous tissue grown in culture for 21 days. Each point represents pooled data from 6 individuals; 3-4 replicas were performed for each individual. Vertical bars represent SEM and are shown when they exceed the symbols in size.

Incubation of rat subcutaneous fibroblasts with histamine (100 μM; n=7) caused a fast (within seconds) $[Ca^{2+}]_i$ rise attaining roughly 30% and 42% of the maximal calcium loads produced by ionomycin (5 μM; 100% response) at culture days 7 and 28, respectively (Figure 11). After the initial rise, intracellular $[Ca^{2+}]_i$ levels were kept fairly constant throughout time, with no decay occurring within the 5-min time of incubation with histamine as indicated by signal-to-noise slopes near 1 (Figure 11).

A



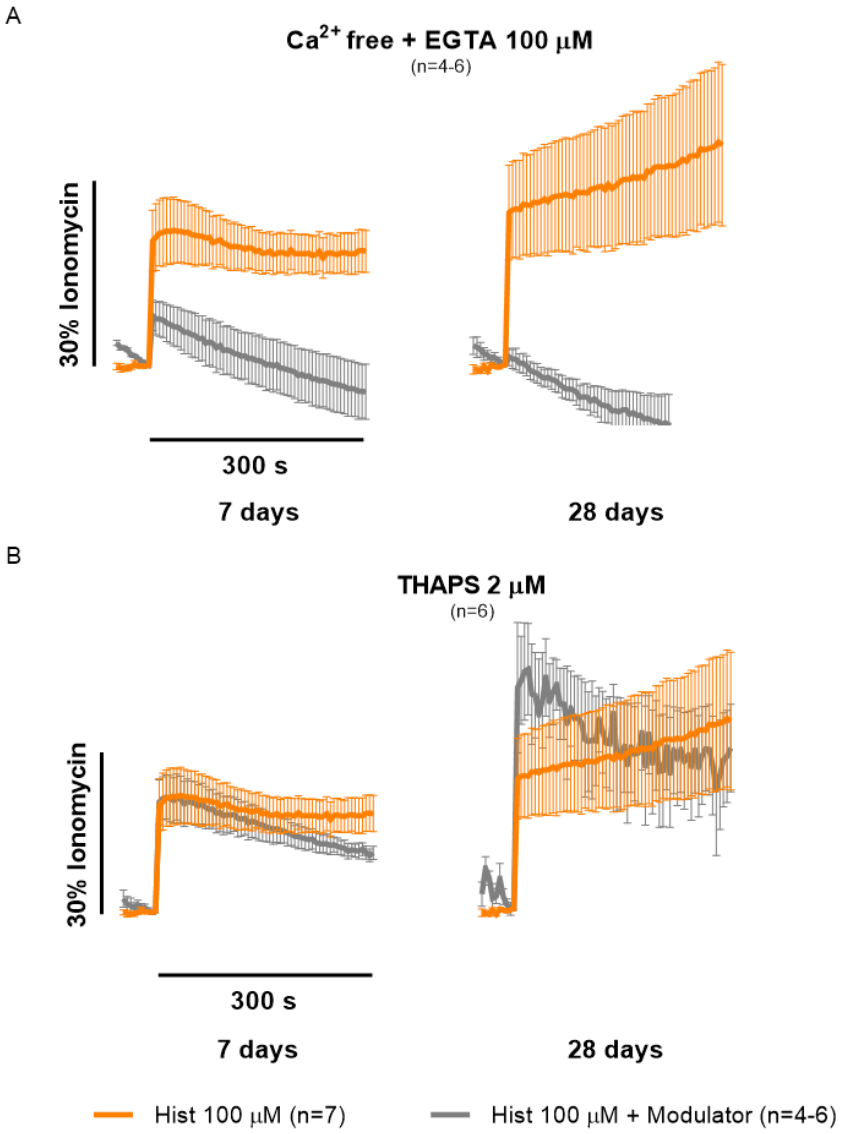
B

Modulator	Slope	
	7d	28d
Hist 100 μM	0.982	1.128

Figure 11 - Histamine (100 μM) produces fast and sustained intracellular Ca^{2+} ($[Ca^{2+}]_i$) rises in rat subcutaneous fibroblasts grown in culture for 7 and 28 days. Cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW; changes in fluorescence were detected in the time-lapse mode using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 μM; 100% response). Shown are $[Ca^{2+}]_i$ oscillations produced by histamine (100 μM). Slopes for each day are depicted in B. Each data set represents pooled data from 7 individuals; 2-3 measurements were made for each individual. Vertical bars represent SEM and are shown when they exceed the lines in size.

Histamine (100 μM)-induced $[Ca^{2+}]_i$ accumulation in rat subcutaneous fibroblasts was prevented ($p<0.05$) by removal of external Ca^{2+} (Ca^{2+}-free medium plus EGTA; 100 μM, n=4-6) (Figure 12A), but the same was not observed after depleting intracellular Ca^{2+} stores with the selective inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin (THAPS 2 μM, n=6) (Figure 12B), both at culture days 7 and 28.

Like that observed in human cells, histamine (100 μM) increased by 6-fold the release of ATP from rat subcutaneous fibroblasts grown in culture for 7 days. This was appreciated measuring the T_3/T_2 ratio in pmol of ATP normalized by the corresponding MTT values (see Methods for details).



C

Modulator	Slope	
	7d	28d
Hist 100 µM	0.982	1.128
EGTA 100 µM	-0.528*	-5.538*
THAPS 2 µM	0.513	0.428

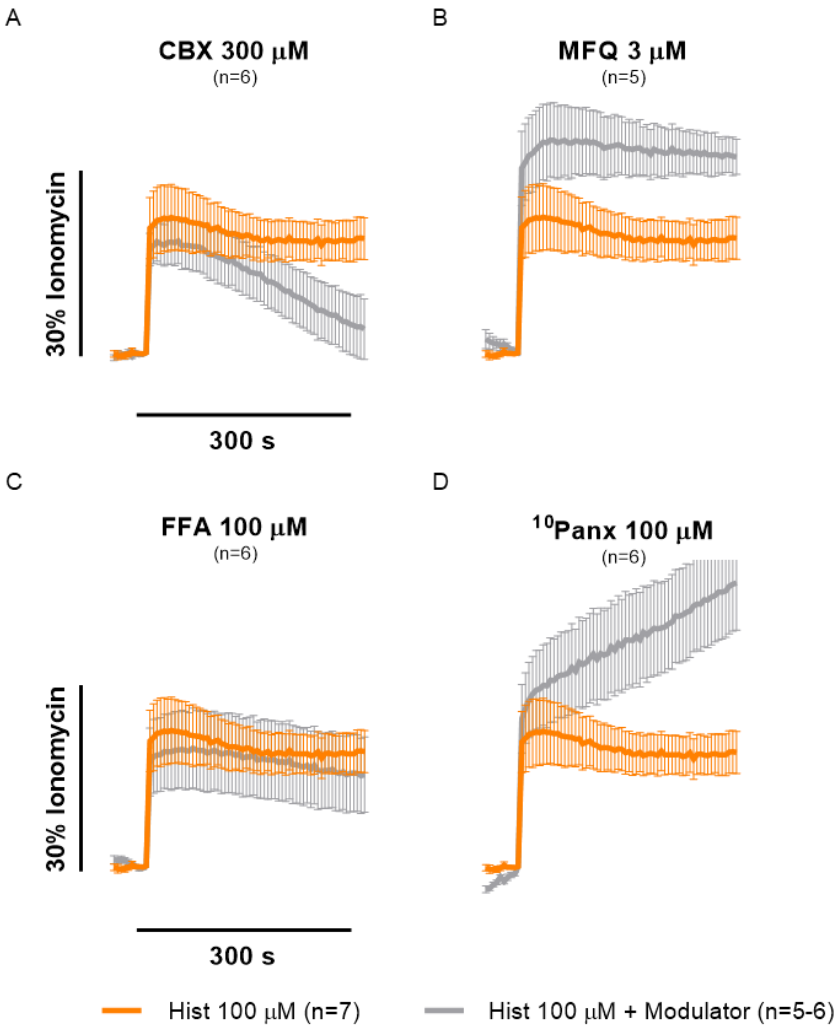
Figure 12 - Histamine-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) accumulation in rat subcutaneous fibroblasts depends on the influx of Ca^{2+} from the extracellular milieu. Cells were allowed to grow in culture for 7 (leftward panels) and 28 (rightward panels) days; then, the cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW and changes in fluorescence were detected in the time-lapse mode using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM; 100% response). Shown are $[Ca^{2+}]_i$ oscillations produced by histamine (100 µM) applied after removal of extracellular Ca^{2+} (Ca^{2+}-free medium plus EGTA 100 µM; A) and in the presence of the selective endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (THAPS, 2 µM; B). The slope of $[Ca^{2+}]_i$ decay for each condition is shown in Table C in comparison to the slope produced by histamine (100 µM) alone. Each point represents pooled data from 4-7 animals; 2-3 measurements were made for each individual. Vertical bars represent SEM and are shown when they exceed the lines in size. * $p<0.05$ (one-way ANOVA) represents significant differences compared to the effect of histamine (100 µM) alone.

5. Connexin-containing hemichannels sensitive to carbenoxolone might play a role in histamine-induced $[Ca^{2+}]_i$ accumulation

Considering that histamine-induced $[Ca^{2+}]_i$ accumulation is sustained by the cooperation with ATP release triggered by the autacoid in human subcutaneous fibroblasts (2), we thought it was interesting to test the action of selective inhibitors of nucleotide-releasing hemichannels on histamine $[Ca^{2+}]_i$ responses.

In the presence of carbenoxolone (CBX; 300 μM, n=6), a non-selective inhibitor of connexins Cx26, Cx30, Cx32, Cx43, and Cx46, that also blocks Panx1-containing hemichannels (46), histamine (100 μM) was unable to sustain intracellular $[Ca^{2+}]_i$ levels in rat subcutaneous fibroblasts grown in culture for 7 days; the decay started approximately 1 min after the initial $[Ca^{2+}]_i$ rise (Figure 13A).

The inhibitory effect of CBX (300 μM) on histamine-induced $[Ca^{2+}]_i$ accumulation was not reproduced by (i) mefloquine (MFQ; 3 μM, n=5), which has been described as completely blocking Cx36- and Cx50-containing hemichannels (79, 80) (Figure 13B), (ii) flufenamic acid (FFA; 100 μM, n=6), which blocks preferentially hemichannels containing Cx43 (81) (Figure 13C), and (iii) the selective Panx1 mimetic inhibitory peptide, ^{10}Panx (100 μM, n=6) (82) (Figure 13D). By yet unknown reason, histamine (100 μM) plus MFQ (3 μM) caused a higher initial $[Ca^{2+}]_i$ rise compared to the autacoid alone (Figure 13B). Unexpectedly, ^{10}Panx (100 μM) progressively increased histamine-induced $[Ca^{2+}]_i$ accumulation from the initial $[Ca^{2+}]_i$ rise onwards (Figure 13D).



E

Modulator	Slope
Hist 100 µM	0.982
CBX 300 µM	-0.078
MFQ 3 µM	1.003
FFA 100 µM	0.673
10Panx 100 µM	1.522

Figure 13 - **Connexin-containing hemichannels sensitive to carbenoxolone might play a role in histamine-induced $[Ca^{2+}]_i$ accumulation.** Cells were allowed to grow in culture for 7 days; then, the cells were pre-incubated with the fluorescent calcium indicator Fluo-4 NW and changes in fluorescence were detected in the time-lapse mode using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM; 100% response). Shown are $[Ca^{2+}]_i$ oscillations produced by histamine (100 µM) applied after pre-incubation with Cxs and Panx1 hemichannels modulators, namely CBX (300 µM, A), MFQ (3 µM, B), FFA (100 µM, C) and ^{10}Panx (100 µM, D). The slope of $[Ca^{2+}]_i$ decay for each condition is shown in Table E in comparison to the slope produced by histamine (100 µM) alone. Each point represents pooled data from 5-7 animals; 2-3 measurements were made for each individual. Vertical bars represent SEM and are shown when they exceed the lines in size.

6. Sustained adenosine A_{2A} receptors activation favors histamine-induced ATP release and intracellular $[Ca^{2+}]_i$ accumulation

Taking into consideration the pro-fibrotic effect of the A_{2A}R agonist, CGS21680, we set to investigate their role on histamine-induced ATP release and intracellular $[Ca^{2+}]_i$ accumulation by rat subcutaneous fibroblasts at culture day 7.

Using the luciferin-luciferase bioluminescence assay, data shows that CGS21680 (30 nM, n=3) ($p>0.05$) increased the amount of histamine (100 μM)-induced ATP release from rat subcutaneous fibroblasts measured as increases in T_3/T_2 ratios in pmol of ATP normalized by the corresponding MTT values (Figure 14). The potentiating effect of CGS21680 (30 nM) was prevented if co-applied with the selective A_{2A}R antagonist, SCH442416 (10 nM) ($p<0.05$) (Figure 14).

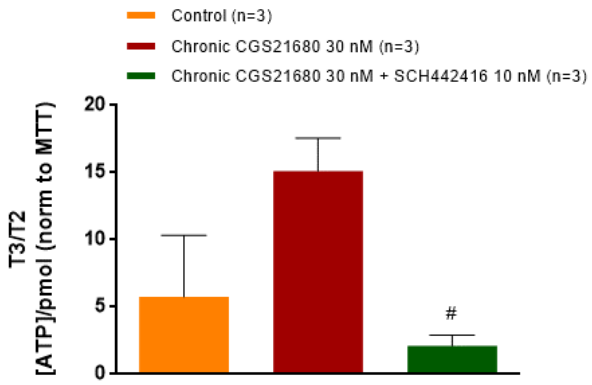
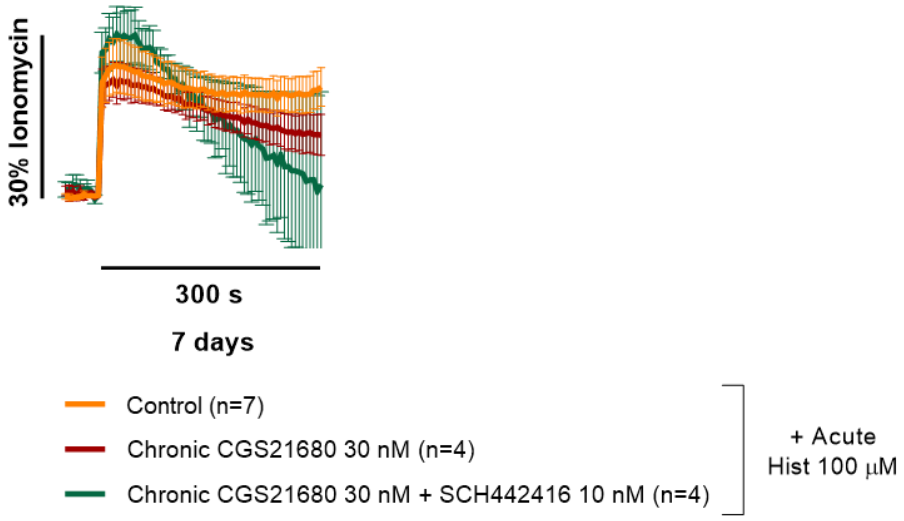


Figure 14 - Sustained adenosine A_{2A} receptors activation favors histamine-induced ATP release in rat subcutaneous fibroblasts grown in culture for 7 days. ATP was measured as the T3/T2 ratio in pmol normalized to MTT content in control conditions (orange) and after treatment of the cells with CGS21680 (30 nM, red) or CGS21680 (30 nM) plus SCH442416 (10 nM, green) for 7 days. Each bar represents the mean ± SEM of 3-4 replicates of 3 animals. # $p<0.05$ (student's t-test) represents significant differences compared to the effect of CGS21680 (30 nM) alone.

Treatment of rat subcutaneous fibroblast cultures for 7 days with the A_{2A}R agonist, CGS21680 (30 nM), did not significantly ($p>0.05$) modify the effect of histamine (100 μM) on intracellular $[Ca^{2+}]_i$ accumulation (Figure 15). Yet, when CGS21680 (30 nM) was used together with the selective A_{2A}R agonist, SCH442416 (10 nM), histamine (100 μM) was unable to sustain the intracellular $[Ca^{2+}]_i$ levels, which started to decay approximately 1 min after the initial $[Ca^{2+}]_i$ rise (Figure 15). This response pattern resembles that observed after pretreatment with the hemichannels blocker, CBX (300 μM) (see Figure 13A).

A



B

Treatment	Slope
Control	0.982
CGS21680 30 nM	0.318
CGS21680 30 nM + SCH442416 10 nM	-0.129

Figure 15 - Effect of A_{2A}R activation on histamine-induced calcium accumulation. Cells were allowed to grow in culture for 7 days either in the absence or in the presence of CGS21680 (30 nM) and CGS21680 30 nM plus SCH442416 10 nM. The cells were, then, pre-incubated with the fluorescent calcium indicator Fluo-4 NW and changes in fluorescence were detected in the time-lapse mode using a microplate reader. $[Ca^{2+}]_i$ transients were calibrated to the maximal calcium load produced by ionomycin (5 µM; 100% response). Shown are $[Ca^{2+}]_i$ oscillations produced by histamine (100 µM) in control conditions (orange, A) and after treatment of the cells with CGS21680 (30 nM, red; A) or CGS21680 (30 nM) plus SCH442416 (10 nM, green; A) for 7 days. The slope of $[Ca^{2+}]_i$ decay for each condition is shown in Table B in comparison to the slope produced by histamine (100 µM) alone on untreated cells. Each line represents the mean ± SEM of 2-3 replicates of 4-7 animals. Statistical analysis was performed using one-way ANOVA.

Discussion and Conclusion

Adenosine is present in most biological fluids. Under basal or physiological conditions, this nucleoside can be found at higher extracellular concentrations when comparing to its intracellular ones mainly due to the catabolism of nucleotides such as ATP or ADP. Under stressful conditions, however, adenosine concentration may increase up to the μM range, suggesting a possible role for this nucleoside in pathophysiological conditions. Amongst numerous other features, an adenosine role in tissue remodeling, often connected to nociception and chronic pain, has emerged (3).

Previous findings from our group demonstrated that the human subcutaneous tissue releases ATP in response to inflammatory mediators, namely bradykinin (1) and histamine (2). Once released, ATP is promptly converted to AMP by NTPDase1/2, which is then dephosphorylated into ADO by Ecto-5'-NT. Mesenchymal originated cells typically exhibit low ability to inactivate ADO, due to their low expression levels of ADA and nucleoside uptake capacity, thus allowing the nucleoside to accumulate in the extracellular milieu, particularly under stressful conditions. These findings, together with the fact that the $A_{2A}R$ and A_3R are highly expressed in fascial fibroblasts ((3), see also Figure 5), prompt the hypothesis that ADO may exert a critical role in tissue remodeling, inflammation and myofascial pain perception.

Using rat subcutaneous fibroblast grown in culture for 21 days, we show here that ADO acting on both $A_{2A}R$ and A_3R increases cell viability and growth (MTT assay), with the $A_{2A}R$ agonist also increasing collagen production (Sirius Red assay) by these cells, at least at culture day 21. The pro-fibrotic effect of CGS21680 was significantly attenuated upon selectively blocking the $A_{2A}R$ receptor with SCH442416. These findings agree with those obtained by other authors suggesting that the $A_{2A}R$ favors fibrosis in the skin, the liver, and the lungs (71). Data are also in accordance with the observations by Herman-de-Sousa *et al* (3), suggesting that adenosine receptors expression in rat subcutaneous fibroblasts does not differ much from those found in the Human. It remains, however, to be explored the dynamics of this adenosine receptor's expression and activation profile in inflammatory conditions and whether it may influence pain perception in these tissues.

Next, we took advantage of the fact that, the pro-inflammatory autacoid, histamine, promoted cell growth/viability, increased intracellular $[Ca^{2+}]_i$ accumulation and ATP release from rat subcutaneous fibroblasts, following a similar activation profile of the

profibrotic effect of ADO, via $A_{2A}R$ activation, in these cells. Histamine-induced intracellular $[Ca^{2+}]_i$ rise was prolonged in time due to endurance of Ca^{2+} influx through the plasma membrane. This was demonstrated because histamine-induced $[Ca^{2+}]_i$ accumulation was fully prevented by removal of Ca^{2+} from the extracellular medium, but not when Ca^{2+} from intracellular reservoirs was depleted by inhibition of the endoplasmic reticulum Ca^{2+}/ATPase by thapsigargin.

Interestingly, 7-day sustained activation of ADO $A_{2A}R$ of rat subcutaneous fibroblasts with CGS21680 increased by 9-fold the release of ATP induced by histamine in comparison to untreated cells, an effect that was fully prevented by selectively blocking these receptors with $A_{2A}R$ antagonist, SCH442416. Likewise, continuous blockage of the $A_{2A}R$ offset the ability of histamine to sustain intracellular $[Ca^{2+}]_i$ levels after the initial fast rise, even when tested in the presence of the $A_{2A}R$ agonist, CGS21680. Surprisingly, the inhibitory effect of the $A_{2A}R$ blockage on histamine-induced intracellular $[Ca^{2+}]_i$ accumulation resembled the effect of carbenoxolone, a non-selective inhibitor of connexins Cx26, Cx30, Cx32, Cx43 and Cx46, also capable of blocking Panx1-containing hemichannels, under similar conditions. The lack of significant effects of mefloquine, flufenamic acid and ^{10}Panx on histamine-induced $[Ca^{2+}]_i$ accumulation rule out the involvement of Cx36, Cx43, Cx50 and Panx1 in this endeavor. Thus, data suggest that adenosine, via $A_{2A}R$ activation, may exert a pro-inflammatory effect by promoting histamine-induced ATP release and intracellular Ca^{2+} signaling endurance. Whether or not $A_{2A}R$ activation affects the nucleotide release via carbenoxolone-sensitive hemichannels in histamine stimulated subcutaneous fibroblasts remains to be investigated in the near future. One cannot, however, disregard the putative participation of other nucleotide releasing mechanisms, such as the vesicular transport and facilitated diffusion by nucleotide through specific ABC transporters and P2X7/P2X4 receptor pores, to the facilitatory effect of adenosine $A_{2A}R$ on histamine-induced ATP release and $[Ca^{2+}]_i$ signaling endurance in rat subcutaneous fibroblasts.

Although not described as a classical $A_{2A}R$ effect, calcium mobilization due to this receptor activation was previously reported on pulmonary endothelial human cells as part of an interplay between phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB), among other kinases, to promote cell growth (83). Despite promoting fibrosis and histamine-mediated pro-inflammatory signals (this study), the idea that selective activation of adenosine $A_{2A}R$ may lead to immunosuppression is widely known, as this receptor has been shown to suppress responses in other immune-mediated diseases, namely in *Myasthenia gravis* (84). It has also been shown that activation of the $A_{2A}R$

favors *in vitro* collagen production both in human and murine models of scleroderma (85), an autoimmune disease affecting several body structures including the skin. The mechanism underlying this feature was further unraveled using human skin fibroblasts. Data suggest that it might involve the adenylyl cyclase/exchange protein activated by cAMP (AC/EPAC) pathway (3, 86), including downstream suppression of the transcription factor Fli1, a constitutive repressor of matrix proteins synthesis, as well as overexpression of the connective tissue growth factor (CTGF) (72). The results hereby presented are promising due to the fact that this receptor often represents a target for inflammatory processes, hopefully including musculoskeletal chronic pain.

In conclusion, data shows that the $A_{2A}R$ exerts pro-fibrotic and pro-inflammatory effects on rat subcutaneous fibroblasts, similarly to that observed in other tissues, including in human subcutaneous fibroblasts. In this context, it was demonstrated for the first time in this study that sustained activation of the adenosine $A_{2A}R$ subtype exerts a synergistic effect with histamine (and, perhaps, with other inflammatory mediators) favoring intracellular $[Ca^{2+}]_i$ endurance and the release of ATP (danger molecule). It remains, however, to be explored the role of the adenosine $A_{2A}R$, (i) on the mechanisms regulating the release of ATP, including the expression and function of carbenoxolone-sensitive hemichannels, and (ii) on the crosstalk between subcutaneous fibroblasts, sensory nerve endings and inflammatory/immune cells invading the subcutaneous tissue under pathological contexts (e.g. dermal fibrosis, fibromyalgia and myofascial pain).

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Annexes

1. IJUP 13th Edition 2020 – Encontro de Investigação Jovem da Universidade do Porto

UNRAVELLING ADENOSINE RECEPTORS SIGNALING IN RAT SUBCUTANEOUS FIBROBLASTS: A NOVEL APPROACH TO MYOFASCIAL PAIN

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Chronic myofascial pain is associated to inflammation-induced subcutaneous tissue disorganization and continuous stimulation of sensory nerves. Subcutaneous fibroblasts release ATP in contact with inflammatory mediators (e.g. bradykinin, histamine) (Pinheiro et al, 2013, Cell Commun Signal 11:70; Pinheiro et al, 2013 J Biol Chem 288:27571). ATP is promptly converted into adenosine by ecto-NTPDases1/2 and ecto-5'-nucleotidase acting in tandem to promote extracellular accumulation of the nucleoside. Adenosine is a powerful modulator of inflammation and neuronal activation, but controversy still exists regarding its role in tissue fibrosis. Understanding the mechanisms by which subcutaneous fibroblasts respond to adenosine and ultimately communicate with sensory neurons may be paramount to discover new drug targets for myofascial pain. Cultured fibroblasts from the rat subcutaneous tissue were monitored for viability/proliferation (MTT assay) and collagen production (Sirius Red assay) for 28 days. Adenosine receptors expression was assessed by immunofluorescence confocal microscopy. Rat subcutaneous fibroblasts exhibit high A_3 receptor amounts, followed by A_{2A} and A_{2B} receptors, with the A_1 receptor being less expressed. The adenosine analogue, NECA (10 and 100 μM, n=3), progressively decreased fibroblast cells growth and collagen production up to day 21 in culture. Selective activation of the A_{2A} receptor with CGS21680 (1 nM, n=3), decreased collagen production from day 14 onwards, with almost no effect on fibroblast cells proliferation. Activation of the A_3 receptor subtype with 2-Cl-IB-MECA (10 and 100 nM, n=4) showed a late pro-fibrotic effect only observed at culture day 28. The predominant anti-fibrotic effect exert by activation of the A_{2A} receptor subtype is promising given to the fact that this receptor also suppresses responses in other immune-mediated conditions (Oliveira et al, 2015, 2015: 460610).

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2. EUGLOH Annual Student Research Conference 2020

UNRAVELLING ADENOSINE RECEPTORS ROLE IN SUBCUTANEOUS FIBROBLASTS AS NOVEL TARGETS AGAINST MYOFASCIAL PAIN

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Chronic myofascial pain is associated to diffuse subcutaneous inflammation, tissue disorganization and sensory nerves stimulation. Subcutaneous fibroblasts release huge amounts of ATP when challenged by inflammatory mediators (Pinheiro et al, 2013 J Biol Chem 288:27571). ATP is rapidly converted into adenosine by ecto-NTPDases1/2 and ecto-5'-nucleotidase. Extracellular adenosine is a powerful modulator of inflammatory and neuronal responses. Understanding the mechanisms by which subcutaneous fibroblasts respond to adenosine and communicate with sensory neurons may be paramount to discover new drug targets for myofascial pain. Using immunofluorescence confocal microscopy, we show here that rat subcutaneous fibroblasts exhibit high A_3 receptor amounts, followed by A_{2A} and A_{2B} receptors, with only minute amounts of the A_1 receptor being detected. The adenosine analogue, NECA (10 and 100 μM, n=3), progressively decreased fibroblast cells growth (MTT assay) and collagen production (Sirius Red assay) up to day 21 of the cells in culture. Selective activation of the A_{2A} receptor with CGS21680 (1 nM, n=3) decreased collagen production from day 14 onwards. Activation of the A_3 receptor subtype with 2-Cl-IB-MECA (10 and 100 nM, n=4) showed a late pro-fibrotic effect only observed at culture day 28. The predominant anti-fibrotic effect exert by activation of the A_{2A} receptor subtype is promising given to the fact that this receptor also suppresses responses in other immune-mediated conditions (Oliveira et al, 2015, 2015: 460610).

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