Adenosine A_{2A} Receptor Agonist, 2-*p*-(2-Carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine Hydrochloride Hydrate, Inhibits Inflammation and Increases Fibroblast Growth Factor-2 Tissue Expression in Carrageenan-Induced Rat Paw Edema

Armando Ialenti,¹ Elisabetta Caiazzo,¹ Silvana Morello, Rosa Carnuccio, and Carla Cicala

Department of Pharmacy, School of Medicine, University of Naples Federico II, Naples, Italy (A.I., E.C., R.C., C.C.) and Department of Pharmacy, University of Salerno, Fisciano, Salerno, Italy (S.M.)

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ABSTRACT

Adenosine is the final product of ATP metabolism, mainly derived from the action of 5'-nucleotidase cleavage of AMP. Cellular production of adenosine is greatly enhanced in inflamed tissues, ischemic tissues, and under hypoxia, where ATP is released from damaged cells. Much evidence has been accumulated on adenosine anti-inflammatory effects mediated through A_{2A} receptor activation; A_{2A} adenosine receptor has also been shown to play a role in matrix deposition and wound healing in a damaged tissue, contributing to dermal tissue protection and repair. Fibroblast growth factor-2 (FGF-2) is a powerful mitogen for fibroblast; it is expressed by several inflammatory cell types and plays a pivotal role in angiogenesis, wound healing, gastric ulcer protection.

Human recombinant FGF-2 has been shown to have anti-inflammatory effects. The purpose of the present work was to investigate on the anti-inflammatory effect of systemic administration of the adenosine A_{2A} agonist 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride hydrate (CGS21680) in the rat model of carrageenan-induced paw edema. We found that CGS21680 inhibits inflammation induced by carrageenan injection into the rat paw, and this effect is associated to the local reduction of cytokine levels and dermal increase of FGF-2 expression. Our results suggest that FGF-2 might be involved in the anti-inflammatory and tissue protective effect due to A_{2A} receptor activation.

Introduction

Adenosine is an endogenous purine nucleoside mainly derived by the breakdown of ATP. Extracellular adenosine accumulates in inflamed tissues, ischemic tissues, and under hypoxia, where ATP is released from damaged cells (Gordon, 1986). Through the interaction with four types of receptors, designed as A₁, A_{2A}, A_{2B}, and A₃, adenosine contributes to the homeostatic regulation of many systems, such as nervous, cardiovascular, renal, gastrointestinal, and immune system (Jacobson and Gao, 2006; Fredholm, 2007; Antonioli et al., 2008a,b). Much evidence has been accumulated both in vitro and in vivo on adenosine anti-inflammatory effects mediated through A_{2A} receptor activation (Palmer and Trevethick, 2008; Antonioli et al., 2013, 2014).

Interestingly, A_{2A} adenosine receptor has also been shown to play a role in matrix deposition and wound healing in a damaged tissue, contributing to dermal tissue protection and repair (Montesinos et al., 1997, 2002; Chan et al., 2006; Cronstein, 2006). Mazzon and coworkers (2011) demonstrated the beneficial effect of systemic A_{2A} agonist administration in the model of collagen-induced arthritis in mice. It has been shown that adenosine A_{2A} receptor also stimulates matrix and collagen production in dermal fibroblasts (Chan et al., 2013).

The dual role of A_{2A} receptor as anti-inflammatory and tissue repairer has driven the attention of several researchers toward the role of this receptor on skin cells (Burnstock et al., 2012). It has already shown that application of the adenosine A_{2A} receptor agonist CGS21680 reduces pressure-induced skin ulcers in rats (Peirce et al., 2001) and inflammation and epidermal hyperplasia in mice following application of phorbol myristate acetate (Arasa et al., 2014), representing a promising therapeutic agent devoid of the atrophic effect of corticosteroids.

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¹A.I. and E.C. contributed equally to this work.

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ABBREVIATIONS: CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride hydrate; FGF-2, fibroblast growth factor-2; FGFR, FGF receptor; HRP, horseradish peroxidase; MPO, myeloperoxidase; NVP-BGJ398, 3-(2,6-dichloro-3,5-dimethoxyphenyl)-1-(6-(4-(4-ethylpiperazin-1-yl)phenylamino)pyrimidin-4-yl)-1-methylurea; PBS, phosphate-buffered saline; TNF- α , tumor necrosis factor alpha; ZM241385, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol.

It is known that FGF-2 is a powerful mitogen for fibroblast; besides fibroblasts, other inflammatory cell types can express FGF-2, including mononuclear phagocytes, CD4⁺, and CD8⁺ T lymphocytes and mast cells (Artuc et al., 1999). FGF-2 exerts its biologic effects by interacting with four members of FGF-receptor (FGFR1–FGFR4), a family of tyrosine kinase receptors and plays a pivotal role in angiogenesis, wound healing, gastric ulcer protection (Bikfalvi et al., 1997; Pohle et al., 1999). Human recombinant FGF-2 has been shown to inhibit croton oil-induced ear swelling and carrageenan-induced paw edema in mice and to reduce peritonitis induced by carrageenan in mice and rats (Hu and Wu, 2001). Moreover, in a murine model of asthma, recombinant FGF-2 has been shown to reduce airway responsiveness, mucus production, and lung inflammation and also to reduce allergen-induced proliferation of T cells (Jeon et al., 2007).

Thus, it is evident that both adenosine, through A_{2A} receptor, and FGF-2 are on the edge of inflammation and tissue regeneration.

In this study, we used the model of rat carrageenan-induced paw edema, an acute model of inflammation that we previously demonstrated to be sensible to the anti-inflammatory effect of CGS21680 (Caiazzo et al., 2016) and that involves inflammation of epidermis and derma to investigate the anti-inflammatory and tissue protective effect of systemic adenosine A_{2A} receptor activation.

We demonstrate that systemic treatment with CGS21680 inhibits inflammation induced by carrageenan injection into the rat paw, and this effect is paralleled by local increase of FGF-2 expression, suggesting that FGF-2 might be involved in the anti-inflammatory and tissue protective effect due to A_{2A} receptor activation.

Materials and Methods

Animals. All experiments were performed on male Wistar rats (180–220 g; Calco (LC), Italy). Rats, slightly anesthetized with enflurane, received in the left hind paw 100 μ l of λ -carrageenan (Sigma-Aldrich S.r.l., Milan, Italy) suspension (1% w/v). Paw volume was measured at the time zero and each hour for 6 hours by a hydroplethysmometer (Ugo Basile, Comerio, Varese, Italy).

Drug Treatments. Rats were randomly assigned to groups of five each and treated, just before edema induction, with the A_{2A} agonist CGS21680 (0.02–2 mg/kg i.p.; Tocris Bioscience, Bristol, UK); with the A_{2A} antagonist, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]-triazin-5-yl-amino]ethyl)phenol (ZM241385 3 and 6 mg/kg i.p.; Tocris Bioscience); with CGS21680 (2 mg/kg i.p.) plus ZM241385 (3 mg/kg i.p.); or with the vehicle (dimethyl sulfoxide; Sigma-Aldrich S.r.l.). The dose of ZM241385 was chosen based on Caiazzo et al. (2016). From different groups of animals, at different times following edema induction, paws were excised, cut, frozen in liquid nitrogen or fixed in 4% (v/v) buffered formalin (Carlo Erba Reagents S.r.l., Cornaredo, Milan, Italy), and stored for further histologic and biochemical analysis.

All procedures were performed according to the Italian and European regulations (DL 26/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health.

Myeloperoxidase Assay. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was measured in the inflamed paws excised after 3 hours from edema induction as previously described (Mullane et al., 1985). At the specified time following injection of carrageenan, tissues were weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide

dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at 20,000 *g*. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine (Sigma-Aldrich S.r.l.) and 0.1 mM H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was determined as the quantity of enzyme degrading 1 mmol of peroxide per minutes at 37°C and was expressed in milliunits per grams of wet tissue. As control, MPO activity was also measured on tissues obtained from contralateral, noninflamed, paws.

Morphologic Analysis. Tissue samples removed as described above were immediately fixed in 4% (v/v) neutral formalin for 48 hours and embedded in paraffin by conventional techniques. For morphologic analysis, 7- μ m-thick sections were stained with hematoxylin and eosin (Carlo Erba Reagents S.r.l.). The sections were analyzed by using a standard light microscope (10 $\times$ objective) and photographed under low power. Images were taken by a Leica DFC320 video camera (Leica, Milan, Italy) connected to a Leica DM RB microscope using the Leica Application Suite software V 4.1.0. 2.7.

Picro Sirius Red Staining. To visualize tissue collagen, Picro Sirius red staining and polarization microscopy were performed. Briefly, the paraffin sections (7 μ m) were dewaxed and rehydrated. The sections were stained with Mayer's hematoxylin to show the nuclei and then incubated with 0.1% (w/v) Sirius Red solution (Sigma-Aldrich) dissolved in saturated picric acid solution for 1 hour at room temperature. After washing with acidified water, slides were alcohol dehydrated, mounted in a resinous medium, and visualized through a light microscope (Leica Microscope) equipped with a digital camera (Leica DFC320). Polarization microscopy images were visualized through a light microscope (Leica Dialux) equipped with a polarization filter (10 $\times$ objective).

Measurement of Tumor Necrosis Factor α and Interleukin-6 Levels in Rat Paws. In tissue sample homogenates, prepared as described below, tumor necrosis factor alpha (TNF- α) and interleukin-6 levels were evaluated by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Results obtained were expressed as picograms of cytokine per milligrams of protein.

Western Blot Analysis. Tissue samples from rats at different time points were defrosted, weighed, and homogenized with a Polytron (three cycles of 10 seconds at maximum speed). To extract proteins, 1 ml of buffer (β -glycerophosphate, 50 mM; sodium orthovanadate, 100 μ M; MgCl₂, 2 mM; EGTA, 1 mM; dithiothreitol, 1 mM; phenylmethylsulfonyl fluoride, 1 mM; aprotinin, 10 μ g/ml; leupeptin, 10 μ g/ml) was added to 100 mg of tissue samples. After centrifugation at 2500 rpm for 30 minutes at 4°C, protein supernatant content was measured by Bradford reagent (Segrate (MI), Italy). Denatured samples (50 μ g) were subjected to electrophoresis on SDS 15 or 12% polyacrylamide gel (for FGF-2 and A_{2A}, respectively) and transferred onto a nitrocellulose membrane (Protran; (Sigma-Aldrich S.r.l., Milan, Italy). Membranes were blocked by incubation with nonfat dry milk (5% w/v) in phosphate-buffered saline (PBS) supplemented with 0.1% (v/v) Tween 20 for 1 hour at room temperature and then incubated with a rabbit polyclonal anti-FGF-2 antibody (1:200 dilution; Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or with a mouse polyclonal anti-A_{2A} antibody (1:1000 dilution; Novus Biologicals, Inc., Littleton, CO) overnight at 4°C on a shaker. Successively, membranes were washed and then incubated with the secondary antibody conjugated with horseradish peroxidase (HRP), anti-rabbit IgG-HRP (1:5000 dilution; Santa Cruz Biotechnology Inc.), or anti-mouse IgG-HRP (1:2000 dilution; Dako, Glostrup, Denmark) for 3 hours at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Milan, Italy). Densitometric values for each band were determined using Quantity One software (Bio-Rad, Milan, Italy). Successively, to confirm the equal protein loading, membranes were stripped and incubated with rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:500 dilution, Santa Cruz Biotechnology Inc.) and subsequently with anti-rabbit IgG-HRP (1:5000 dilution, Santa Cruz Biotechnology Inc.), both for 3 hours at room temperature.

Immunofluorescence Analysis. Seven micrometer sections of paraffin-embedded paw tissue were deparaffinized, rehydrated, and heat induced for antigen retrieval by using Diva decloaker (Biocar Medical, Casablanca, Morocco). After blocking of endogenous peroxidase (0.3% H₂O₂, 30 minutes) and unspecific binding with avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), sections were incubated with rabbit polyclonal anti-FGF-2 (1:100 dilution; Santa Cruz Biotechnology Inc.) antibody in casein plus Triton X-100 0.3% overnight at 4°C. The next day, sections were washed with PBS plus Tween 0.05% before incubation with fluorescein isothiocyanate-conjugated antirabbit antibody (1:250 dilution; Jackson Immuno Research Laboratories, Inc., West Grove, PA) for 30 minutes at room temperature. Slides were then washed in PBS-Tween 0.05% and mounted in Vectashield Mounting Medium with 4', 6-diamidino-2-phenylindole (1.5 µg/ml; Vector Laboratories). Images were observed using a fluorescence microscope Leica DM RB (Leica Microsystems, Wetzlar, Germany) equipped with appropriate standard filter and acquired using Leica Application Suite V 4.1.0 software.

Inhibition of FGF-2 Signaling. The effect of the A_{2A} agonist CGS21680 (2 mg/kg i.p.) was also evaluated in groups of rats treated with the FGFR inhibitor, 3-(2,6-dichloro-3,5-dimethoxyphenyl)-1-(6-(4-(4-ethylpiperazin-1-yl) phenylamino) pyrimidin-4-yl)-1-methylurea (NVP-BGJ398, 30 mg/kg i.p.; Adipogen, San Diego, CA; Guagnano et al., 2011) or with an anti FGF-2 neutralizing antibody (clone bFM-1), 10 µg/paw (Merck Millipore, Vimodrone (MI), Italy; Even-Chen et al., 2017). All groups received the same amount of dimethyl sulfoxide that was the vehicle for CGS21680 and NVP-BGJ398. Anti FGF-2 neutralizing antibody (10 µg) diluted in distilled water was contained in 100 µl of carrageenan and injected into the paw.

Statistical Analysis. All results were expressed as mean ± S.E.M. and analyzed by one-way analysis of variance followed by Dunnett's or Bonferroni's test for multiple comparisons, as appropriate. A value of $P < 0.05$ was taken as statically significant.

Results

Effect of CGS21680 Treatment on Carrageenan-Induced Edema. Injection of carrageenan in the rat hind paw caused an edema peaking between 3 and 4 hours. Preliminary experiments showed that treatment with CGS21680 inhibited, in a dose-related manner, edema development, and MPO activity in the rat paw (Fig. 1, A and B), on the basis the dose of 2 mg/kg of CGS21680, was chosen for the entire study. The anti-inflammatory effect of CGS21680 (2 mg/kg i.p.) was reversed by coadministration with the A_{2A} antagonist ZM241385 (3 mg/kg i.p.; Fig. 1C). On the contrary, ZM241385 (3 and 6 mg/kg i.p.) alone did not modify edema (data not shown).

Morphologic Analysis. Morphologic examination of tissue sections stained with hematoxylin and eosin showed reduced cell infiltrate in paws from CGS21680 (2 mg/kg i.p.) treated rats; furthermore, in the same samples, there was no sign of edema in the dermal layer and it was evident that the cytoarchitecture of epidermis was intact, with dermal papillae well evident (Fig. 2C), compared with samples obtained by vehicle injected rats, characterized by flattening of epidermal dermal junction in inflamed tissue, suggesting a reduced epidermal dermal cohesion (Fig. 2B).

Picro Sirius Staining. Picro Sirius red staining of inflamed tissue to highlight collagen fibers showed that in tissue sections from inflamed animals dermal collagen fibers were loose (Fig. 2, E and H) compared with paws from control (Fig. 2, D and G) and from CGS21680 (Fig. 2, F and I) treated animals where dermal elastic fibers were well organized.

Fig. 1. Effect of CGS21680 (0.02–2 mg/kg i.p.) on carrageenan-induced rat paw edema and tissue MPO activity. Administration of CGS21680 (0.02–2 mg/kg i.p.) inhibited carrageenan-induced paw edema (A) and MPO activity (B) in inflamed rat paws. The effect of CGS21680 (2 mg/kg i.p.) was reversed by cotreatment with the A_{2A} antagonist ZM241385 (3 mg/kg i.p.) (C). Values are expressed as mean ± S.E.M. ($n = 10$ for each group). Statistical significance was assessed by analysis of variance followed by Dunnett's test; * $P < 0.05$; ** $P < 0.01$ vs. vehicle [dimethyl sulfoxide (DMSO)]. *** $P < 0.01$ vs. control (Ctrl; not injected paw).

Fig. 2. Effect of CGS21680 (2 mg/kg i.p.) on morphologic changes and collagen content in rat paw 3 hours after carrageenan injection. Morphologic evaluation of rat paws was performed by hematoxylin and eosin staining (H&E). (B) Paws from vehicle-treated rats show dermal edema, increased cell infiltration, and flattening of basal lamina (see arrows). (C) Systemic treatment with CGS21680 protects from morphologic changes (see arrows). Original magnification, 10 $\times$; scale bar, 200 μ m. Picro Sirius red staining and polarizing microscopy were performed to visualize tissue collagen in rat paw. Tissue obtained from CGS21680-treated rats (F and I) show compact collagen fibers (see arrow) compared with tissues from vehicle-injected rats (E and H). Original magnification, 10 $\times$; scale bar, 200 μ m.

Cytokine Levels. TNF- α and interleukin-6 levels into the inflamed tissues were significantly reduced by rat treatment with CGS21680 (2 mg/kg i.p.). This effect was reversed by coadministration of ZM241385 (3 mg/kg i.p.) (Fig. 3).

FGF-2 and A_{2A} Expression. The time course of FGF-2 expression in rat paws showed a strong increased expression in the group treated with CGS21680, and the effect was evident at 3 and 4 hours following edema induction (Fig. 4, B and D). Conversely, in the vehicle-treated group, the slight increase in FGF-2 expression, observed at 1 and 2 hours following carrageenan injection, returned to control value at

3 and 4 hours when the inflammation peaked (Fig. 4, A and D). The time course of A_{2A} expression showed an increased protein expression peaking between 3 and 4 hours (Fig. 5, A and D). This increased A_{2A} expression was reduced to control values by CGS21680 (Fig. 5, B and D). Rat systemic treatment with CGS21680 (2 mg/kg i.p.) plus ZM241385 (3 mg/kg i.p.) reversed the effect of administration of CGS21680 alone on both FGF-2 and A_{2A} tissue expression (Fig. 4; Fig. 5, C and D).

Immunofluorescence Analysis for FGF-2. On tissue obtained as described above, FGF-2 (18 kDa) was localized by immunofluorescence analysis. Immunolocalization of FGF-2

Fig. 3. Effect of systemic administration of CGS21680 (2 mg/kg i.p.) on interleukin (IL)-6 and TNF- α content in rat inflamed paws 3 hours after carrageenan injection. CGS21680 reduced both IL-6 (A) and TNF- α (B) content in paw tissue and this effect was reversed by coadministration with the A_{2A} antagonist ZM241385 (3 mg/kg i.p.). Values are expressed as mean $\pm$ S.E.M. (n = 6 for each group). Statistical significance was assessed by analysis of variance followed by Dunnett's test; * P < 0.05; *** P < 0.001 vs. vehicle (DMSO).

Fig. 4. Representative Western blotting (A–C) and densitometric analysis (D) of the time course of FGF-2 expression in rat paws. Treatment with CGS21680 (2 mg/kg i.p.) strongly increased FGF-2 expression in rat paws at 3 and 4 hours after carrageenan injection (B and D). Treatment with ZM241385 (3 mg/kg i.p.) reversed the effect of CGS21680 (C and D). Values are expressed as mean $\pm$ S.E.M. of $n = 5$. Statistical significance was assessed by analysis of variance followed by Bonferroni's test. [#] $P < 0.05$; ^{##} $P < 0.01$; ^{###} $P < 0.001$ vs. Ctrl ^{***} $P < 0.001$ vs. vehicle (DMSO).

was increased in derma layer of paws from CGS21680 treated rats compared with control paws (Fig. 6).

Inhibition of FGF-2 Signaling. Systemic administration to rats of FGFR inhibitor NVP-BGJ398 (30 mg/kg i.p.) or local administration of the anti-FGF-2 neutralizing antibody (10 μ g/paw) reversed the anti-inflammatory effect of CGS21680. Both treatments did not have any effect on edema development (Fig. 7).

Discussion

Carrageenan-induced rat paw edema is a classic model of acute inflammation, widely used to identify new therapeutic targets and to test the anti-inflammatory potential of new molecules. This model has been largely characterized; local changes of epidermis and derma following subcutaneous injection of carrageenan in the hind paw have also been described (Vinegar et al., 1987; Morris, 2003).

Adenosine A_{2A} receptor has been described to be anti-inflammatory in several models of acute and chronic inflammation (Palmer and Trevethick, 2008; Antonioli et al., 2014) and to be involved in adenosine-mediated matrix production and wound healing (Valls et al., 2009). Several studies have reported the beneficial effect of topical application of A_{2A} agonist on injured skin (Montesinos et al., 2002; Arasa et al., 2014); however, the mechanism at the basis is still unclear. However, there is also evidence that under pathologic conditions or following skin physical injury, such as radiation induced injury, A_{2A} receptor activation is responsible for fibrosis (Perez-Aso et al., 2016). Here, we show that systemic administration of A_{2A} agonist CGS21680 to rats inhibits paw

inflammation induced by subcutaneous injection of carrageenan, increases FGF-2 dermal expression, and ameliorates skin cytoarchitecture.

First, we confirmed the functional role of A_{2A} in the control of inflammation, by animal treatment with the A_{2A} agonist CGS21680 or with the antagonist ZM241385. As previously demonstrated (Caiazzo et al., 2016), we found that systemic treatment with A_{2A} agonist CGS21680 prevented edema development, whereas the antagonist ZM241385 did not have any effect (data not shown). Furthermore, CGS21680 inhibitory effect was prevented by coadministration with ZM241385. This finding confirms that the effect of CGS21680 was specific, through A_{2A} receptor stimulation; nonetheless, A_{2A} activation by endogenous adenosine seems not to offer protection against acute inflammation, because A_{2A} antagonism did not exacerbate edema. This finding might suggest that CGS21680 would act through an indirect mechanism, involving stimulation of A_{2A} receptor probably distant from the site of injury and activating anti-inflammatory mechanisms. A similar result was observed by Peirce et al. (2001), who demonstrated that systemic administration of ALT-146e, a selective A_{2A} agonist, reduced skin ulceration induced by recurrent ischemia reperfusion in rats. The effect was reversed by the antagonist ZM241385; however, the antagonist alone did not exacerbate skin ulceration. Authors conclude that the A_{2A} agonist probably acted outside the ischemic zone.

We also focused on skin morphologic changes following subcutaneous injection of carrageenan and found diffuse dermal edema paralleled by evident changes in epidermis cytoarchitecture that was absent following treatment with

Fig. 5. Representative Western blotting (A–C) and densitometric analysis (D) of the time course of A_{2A} expression in rat paws. A_{2A} expression increased at 3 and 4 hours after carrageenan injection (A and D) and it was reduced following rat treatment with CGS 21680 (2 mg/kg i.p.) (B and D). Treatment with ZM241385 (3 mg/kg i.p.) reversed the effect of CGS21680 (C and D). Values are expressed as mean $\pm$ S.E.M. of $n = 5$. Statistical significance was assessed by analysis of variance followed by Bonferroni's test. $^{\#}P < 0.05$; $^{###}P < 0.01$ vs. Ctrl (not injected paw) and $^{***}P < 0.001$ vs. vehicle (DMSO).

CGS21680. Furthermore, Picro Sirius red staining evidenced that dermal collagen fibers were more compact and allineated following rat treatment with CGS21680. It has been demonstrated that adenosine A_{2A} receptor activation stimulates fibroblasts to produce collagen (Perez-Aso et al., 2013), and topical application of A_{2A} agonist facilitates wound closure (Montesinos et al., 1997, 2002). These findings outline the importance of A_{2A} receptor in matrix production, although the mechanism at the basis of A_{2A} receptor protective effect on inflammation and tissue destruction is still unclear. It is known that extracellular matrix and fibroblast, as well as epidermal keratinocytes, are actively involved in the modulation of inflammation (Gilroy et al., 2004; Sorokin, 2010). Scheibner and co-workers (2009) demonstrated an anti-inflammatory role of A_{2A} receptor through modulation of components of extracellular matrix. The same authors also demonstrate that transgenic mice, lacking A_{2A} receptor, are more susceptible to bleomycin-induced lung fibrosis (Scheibner et al., 2009); this latter finding is in contrast with other papers demonstrating that lack of A_{2A} receptor protect from bleomycin-induced fibrosis (Katebi et al., 2008) and that adenosine deaminase-deficient mice, a model of elevated tissue adenosine, develop spontaneous pulmonary and skin fibrosis (Fernández et al., 2008). It is conceivable that A_{2A} receptor plays a different role on tissue regeneration or fibrosis depending on the duration of its stimulation by endogenous adenosine under acute or chronic tissue damage (Luo et al., 2016).

It is known that matrix can act as a reservoir for several growth factors, including fibroblast growth factor-2. FGF-2 is a molecule with autocrine and paracrine functions. Produced by several cell types and released by shedding vesicles (Taverna et al., 2008), FGF-2 plays a pivotal role in angiogenesis, wound healing, gastric ulcer protection, cell proliferation, and inflammation (Bikfalvi et al., 1997; Pohle et al., 1999). FGF-2 is a growth factor stimulating the production of collagen, the major constituent of extracellular matrix also actively involved in resolution of inflammation (Burgess, 2009). Experiments performed in Fgf-2 knockout mice have demonstrated that FGF-2 is required for epithelial repair and resolution of inflammation following intranasal instillation of bleomycin, but it is not required for pulmonary fibrosis (Guzy et al., 2015), according with previous data demonstrating a role for FGF-2 in epidermal repair (Ortega et al., 1998). Here, results obtained by Western blot analysis showed that increased tissue FGF-2 expression following rat treatment with CGS21680 compared with vehicle and even to control, not inflamed, paws and that coadministration with the A_{2A} antagonist ZM241385 reversed the anti-inflammatory effect of CGS21680 and the increase in FGF-2 expression. This finding confirms that these effects are dependent upon adenosine A_{2A} receptor activation. The functional role of FGF-2 signaling in the anti-inflammatory effect of adenosine A_{2A} receptor activation was proved by evidence that NGJ-BVP398, a pan-specific FGFR inhibitor (Guagnano et al., 2011), reversed the effect of CGS21680. This result might also

Fig. 6. Immunofluorescence detection of FGF-2 on rat paws excised 3 hours after carrageenan injection. Increased immunostaining for FGF-2 (green) was localized in derma of paws from CGS21680-treated rats (2 mg/kg i.p.) (C). Cell nuclei (blue) were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Original magnification, 20 $\times$; scale bar, 100 μ m. (A) Ctrl; (B) vehicle.

be suggestive of a physical interaction between adenosine A_{2A} receptor and FGFR, as already demonstrated in vitro (Flajolet et al., 2008). In the end, evidence that the anti-inflammatory effect of systemic administration of CGS21680 was also reversed by the local injection into the paw of the neutralizing anti-FGF-2 further links the increase in tissue FGF-2 expression to the anti-inflammatory effect mediated by A_{2A} activation. We also evaluated A_{2A} receptor expression in rat inflamed paws to evaluate whether there was correspondence with FGF-2 expression. Results obtained by Western blot analysis showed that A_{2A} receptor protein expression increased in the inflamed tissue rapidly following edema induction, with a peak at 3 to 4 hours, returning to control value 6 hours thereafter. Further analysis showed that the

increased A_{2A} expression was reduced following treatment with CGS21680. This finding would have suggested that the increased A_{2A} expression in inflamed tissues might reflect cell, likely neutrophils, accumulation, and also considered that it was paralleled by an increased MPO activity and reduced by CGS21680 treatment.

In conclusion, we demonstrate that the adenosine A_{2A} receptor agonist CGS21680 systemically administered to rats prevents paw edema development and cytokine increase in the rat paw injected with carrageenan by way of a mechanism dependent by receptor activation, because both effects are inhibited by the antagonist ZM241385. At the same time, A_{2A} agonist cause an increased expression of FGF-2 into the rat paw that is also dependent upon receptor activation and likely

Fig. 7. Effect of NVP-BJG398 (30 mg/kg i.p.) and of anti-FGF-2 neutralizing antibody (10 μ g/paw) on carrageenan-induced rat paw edema. Both NVP-BJG398 (A) and anti-FGF-2 neutralizing antibody (B) reversed the anti-inflammatory effect of CGS21680 (2 mg/kg i.p.) and did not modify edema development. Values are expressed as mean $\pm$ S.E.M. ($n = 5$ for each group). Statistical significance was assessed by analysis of variance followed by Bonferroni's test; (A) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. vehicle (DMSO). # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. CGS + NVP-BJG398 (A) or vs. CGS + anti-FGF-2 (B).

related to the anti-inflammatory effect. Our results shed light on the role of adenosine A_{2A} receptor in the modulation of an innate immune response and link its tissue protective effect to an increased expression of FGF-2.

Authorship Contributions

Participated in research design: Ialenti, Caiazzo, Cicala.

Conducted experiments: Caiazzo, Morello, Cicala.

Performed data analysis: Ialenti, Caiazzo, Morello, Cicala.

Wrote or contributed to the writing of the manuscript: Ialenti, Caiazzo, Carnuccio, Cicala.

References

- Antonoli L, Blandizzi C, Pacher P, and Haskó G (2013) Immunity, inflammation and cancer: a leading role for adenosine. *Nat Rev Cancer* **13**:842–857.
- Antonoli L, Csóka B, Fornai M, Colucci R, Kóka E, Blandizzi C, and Haskó G (2014) Adenosine and inflammation: what's new on the horizon? *Drug Discov Today* **19**: 1051–1068.
- Antonoli L, Fornai M, Colucci R, Ghisu N, Tuccori M, Del Tacca M, and Blandizzi C (2008a) Pharmacological modulation of adenosine system: novel options for treatment of inflammatory bowel diseases. *Inflamm Bowel Dis* **14**:566–574.
- Antonoli L, Fornai M, Colucci R, Ghisu N, Tuccori M, Del Tacca M, and Blandizzi C (2008b) Regulation of enteric functions by adenosine: pathophysiological and pharmacological implications. *Pharmacol Ther* **120**:233–253.
- Arasa J, Martos P, Terencio MC, Valcende-Cavero F, and Montesinos MC (2014) Topical application of the adenosine A_{2A} receptor agonist CGS-21680 prevents phorbol-induced epidermal hyperplasia and inflammation in mice. *Exp Dermatol* **23**:555–560.
- Artuc M, Hermes B, Steckelings UM, Grützkau A, and Henz BM (1999) Mast cells and their mediators in cutaneous wound healing—active participants or innocent bystanders? *Exp Dermatol* **8**:1–16.
- Bikfalvi A, Klein S, Pintucci G, and Rifkin DB (1997) Biological roles of fibroblast growth factor-2. *Endocr Rev* **18**:26–45.
- Burgess JK (2009) The role of the extracellular matrix and specific growth factors in the regulation of inflammation and remodelling in asthma. *Pharmacol Ther* **122**: 19–29.
- Burnstock G, Knight GE, and Greig AV (2012) Purinergic signaling in healthy and diseased skin. *J Invest Dermatol* **132**:526–546.
- Caiazzo E, Maione F, Morello S, Lapucci A, Paccosi S, Steckel B, Lavecchia A, Parenti A, Iuvone T, Schrader J, et al. (2016) Adenosine signalling mediates the anti-inflammatory effects of the COX-2 inhibitor nimesulide. *Biochem Pharmacol* **112**: 72–81.
- Chan ES, Fernandez P, Merchant AA, Montesinos MC, Trzaska S, Desai A, Tung CF, Khos DN, Pillinger MH, Reiss AB, et al. (2006) Adenosine A_{2A} receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum* **54**:2632–2642.
- Chan ES, Liu H, Fernandez P, Luna A, Perez-Aso M, Bujor AM, Trojanowska M, and Cronstein BN (2013) Adenosine A_{2A} receptors promote collagen production by a Fli1- and CTGF-mediated mechanism. *Arthritis Res Ther* **15**:R58.
- Cronstein BN (2006) Adenosine receptors and wound healing, revised. *Sci World J* **6**: 984–991.
- Even-Chen O, Sadot-Sogrin Y, Shaham O, and Barak S (2017) Fibroblast growth factor 2 in the dorsomedial striatum is a novel positive regulator of alcohol consumption. *J Neurosci* **37**:8742–8754.
- Fernández P, Trzaska S, Wilder T, Chiriboga L, Blackburn MR, Cronstein BN, and Chan ES (2008) Pharmacological blockade of A_{2A} receptors prevents dermal fibrosis in a model of elevated tissue adenosine. *Am J Pathol* **172**:1675–1682.
- Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnon N, Bendor J, Wallach I, Nairn AC, Surmeier DJ, and Greengard P (2008) FGF acts as a co-transmitter through adenosine A_(2A) receptor to regulate synaptic plasticity. *Nat Neurosci* **11**: 1402–1409.
- Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* **14**:1315–1323.
- Gilroy DW, Lawrence T, Perretti M, and Rossi AG (2004) Inflammatory resolution: new opportunities for drug discovery. *Nat Rev Drug Discov* **3**:401–416.
- Gordon JL (1986) Extracellular ATP: effects, sources and fate. *Biochem J* **233**: 309–319.
- Guagnano V, Furet P, Spanka C, Bords V, Le Douget M, Stamm C, Brueggen J, Jensen MR, Schnell C, Schmid H, et al. (2011) Discovery of 3-(2,6-dichloro-3,5-dimethoxy-phenyl)-1-6-[4-(4-ethyl-piperazin-1-yl)-phenylamino]-pyrimidin-4-yl-1-methyl-urea (NVP-BGJ398), a potent and selective inhibitor of the fibroblast growth factor receptor family of receptor tyrosine kinase. *J Med Chem* **54**: 7066–7083.
- Guzy RD, Stoilov I, Elton TJ, Mecham RP, and Ornitz DM (2015) Fibroblast growth factor 2 is required for epithelial recovery, but not for pulmonary fibrosis, in response to bleomycin. *Am J Respir Cell Mol Biol* **52**:116–128.
- Hu YF and Wu YJ (2001) Effect of recombinant human basic fibroblast growth factor on acute inflammation in mice and rats. *Acta Pharmacol Sin* **22**:375–379.
- Jacobson KA and Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5**:247–264.
- Jeon SG, Lee CG, Oh MH, Chun EY, Gho YS, Cho SH, Kim JH, Min KU, Kim YY, Kim YK, et al. (2007) Recombinant basic fibroblast growth factor inhibits the airway hyperresponsiveness, mucus production, and lung inflammation induced by an allergen challenge. *J Allergy Clin Immunol* **119**:831–837.
- Katebi M, Fernandez P, Chan ES, and Cronstein BN (2008) Adenosine A_{2A} receptor blockade or deletion diminishes fibrocyte accumulation in the skin in a murine model of scleroderma, bleomycin-induced fibrosis. *Inflammation* **31**:299–303.
- Luo F, Le NB, Mills T, Chen NY, Karmouty-Quintana H, Molina JG, Davies J, Philip K, Volcik KA, Liu H, et al. (2016) Extracellular adenosine levels are associated with the progression and exacerbation of pulmonary fibrosis. *FASEB J* **30**: 874–883.
- Mazzon E, Esposito E, Impellizzeri D, DI Paola R, Melani A, Bramanti P, Pedata F, and Cuzzocrea S (2011) CGS 21680, an agonist of the adenosine (A_{2A}) receptor, reduces progression of murine type II collagen-induced arthritis. *J Rheumatol* **38**: 2119–2129.
- Montesinos MC, Desai A, Chen JF, Yee H, Schwarzschild MA, Fink JS, and Cronstein BN (2002) Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A_(2A) receptors. *Am J Pathol* **160**:2009–2018.
- Montesinos MC, Gadangi P, Longaker M, Sung J, Levine J, Nilsen D, Reibman J, Li M, Jiang CK, Hirschhorn R, et al. (1997) Wound healing is accelerated by agonists of adenosine A₂ (G alpha s-linked) receptors. *J Exp Med* **186**:1615–1620.
- Morris CJ (2003) Carrageenan-induced paw edema in the rat and mouse. *Methods Mol Biol* **225**:115–121.
- Mullane KM, Kraemer R, and Smith B (1985) Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* **14**:157–167.
- Ortega S, Ittmann M, Tsang SH, Ehrlich M, and Basilico C (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc Natl Acad Sci USA* **95**:5672–5677.
- Palmer TM and Trevelthick MA (2008) Suppression of inflammatory and immune responses by the A_(2A) adenosine receptor: an introduction. *Br J Pharmacol* **153** (Suppl 1):S27–S34.
- Peirce SM, Skalak TC, Rieger JM, Macdonald TL, and Linden J (2001) Selective A_(2A) adenosine receptor activation reduces skin pressure ulcer formation and inflammation. *Am J Physiol Heart Circ Physiol* **281**:H67–H74.
- Perez-Aso M, Mediero A, and Cronstein BN (2013) Adenosine A_{2A} receptor (A_{2A}R) is a fine-tune regulator of the collagen1:collagen3 balance. *Purinergic Signal* **9**: 573–583.
- Perez-Aso M, Mediero A, Low YC, Levine J, and Cronstein BN (2016) Adenosine A_{2A} receptor plays an important role in radiation-induced dermal injury. *FASEB J* **30**: 457–465.
- Pohle T, Shahin M, Domschke W, and Konturek JW (1999) Effect of basic fibroblast growth factor on gastric ulcer healing and its own mRNA expression. *Aliment Pharmacol Ther* **13**:1543–1551.
- Scheibner KA, Boodoo S, Collins S, Black KE, Chan-Li Y, Zarek P, Powell JD, and Horton MR (2009) The adenosine a_{2a} receptor inhibits matrix-induced inflammation in a novel fashion. *Am J Respir Cell Mol Biol* **40**:251–259.
- Sorokin L (2010) The impact of the extracellular matrix on inflammation. *Nat Rev Immunol* **10**:712–723.
- Taverna S, Rigogliuso S, Salamone M, and Vittorelli ML (2008) Intracellular trafficking of endogenous fibroblast growth factor-2. *FEBS J* **275**:1579–1592.
- Valls MD, Cronstein BN, and Montesinos MC (2009) Adenosine receptor agonists for promotion of dermal wound healing. *Biochem Pharmacol* **77**:1117–1124.
- Vinegar R, Truax JF, Selph JL, Johnston PR, Venable AL, and McKenzie KK (1987) Pathway to carrageenan-induced inflammation in the hind limb of the rat. *Fed Proc* **46**:118–126.

Address correspondence to: Dr. Carla Cicala, Department of Pharmacy, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy. E-mail: cicala@unina.it