Purinergic Signalling (2013) 9:573–583
DOI 10.1007/s11302-013-9368-1

ORIGINAL ARTICLE

Adenosine A2A receptor ($A_{2A}R$) is a fine-tune regulator of the collagen1:collagen3 balance

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Received: 2 April 2013 / Accepted: 15 May 2013 / Published online: 8 June 2013

Abstract Adenosine is a potent endogenous anti-inflammatory and immunosuppressive metabolite that is a potent modulator of tissue repair. However, the adenosine A_{2A} receptor ($A_{2A}R$)-mediated promotion of collagen synthesis is detrimental in settings such as scarring and scleroderma. The signaling cascade from $A_{2A}R$ stimulation to increased collagen production is complex and obscure, not least because cAMP and its downstream molecules PKA and Epac1 have been reported to inhibit collagen production. We therefore examined $A_{2A}R$-stimulated signaling for collagen production by normal human dermal fibroblasts (NHDF). Collagen1 (Col1) and collagen3 (Col3) content after $A_{2A}R$ activation by CGS21680 was studied by western blotting. Contribution of PKA and Epac was analyzed by the PKA inhibitor PKI and by knockdowns of the PKA-Cα, -Cβ, -Cγ, Epac1, and Epac2. CGS21680 stimulates Col1 expression at significantly lower concentrations than those required to stimulate Col3 expression. $A_{2A}R$ stimulates Col1 expression by a PKA-dependent mechanism since PKA inhibition or PKA-Cα and -Cβ knockdown prevents $A_{2A}R$-mediated Col1 increase. In contrast, $A_{2A}R$ represses Col3 via PKA but stimulates both Col1 and Col3 via an Epac2-dependent mechanism. $A_{2A}R$ stimulation with CGS21680 at 0.1 μM increased Col3 expression only upon PKA blockade. $A_{2A}R$ activation downstream signaling for Col1 and Col3 expression proceeds via two distinct pathways with varying sensitivity to cAMP activation; more highly cAMP-sensitive PKA activation stimulates Col1 expression, and less cAMP-sensitive Epac activation promotes both Col1 and Col3 expression. These observations may explain the dramatic change in Col1:Col3 ratio in hypertrophic and immature scars, where adenosine is present in higher concentrations than in normal skin.

Keywords Adenosine · Adenosine receptor A2 · Collagen type I · Collagen type III · PKA · Epac

Abbreviations

Col1	Collagen type I
Col3	Collagen type III
$A_{2A}R$	Adenosine A2 receptor
NHDF	Normal human dermal fibroblasts

Introduction

Adenosine, a product of ATP catabolism [1], is present in most biological fluids and is elevated during tissue or organ stress when it acts as a potent endogenous modulator of inflammation and tissue repair [2, 3]. Under basal conditions, the extracellular adenosine concentration is constant (30–300 nM). In contrast, its concentration increases dramatically to micromolar ranges when there is increased ATP catabolism, as in tissue or cellular necrosis and hypoxia [4]. It has been previously reported that adenosine, acting via adenosine 2A receptor ($A_{2A}R$), promotes wound healing [5] and excisional wound closure in both normal and diabetic mice, and the enhancement in dermal wound healing is accompanied by an increase in matrix (collagen) in the wounds [6, 7]. In addition, the $A_{2A}R$ plays an important role in the pathogenesis of fibrotic malignancies of the skin such as dermal fibrosis and scleroderma [8–10] and liver [11]. Thus, blockade or deletion of adenosine $A_{2A}R$ prevents dermal fibrosis in mice treated with bleomycin [12], and $A_{2A}R$ blockade prevents scarring by reducing collagen content and misalignment [10]. Similarly, deletion or blockade of adenosine $A_{2A}R$ prevents liver fibrosis in mice treated with CCl_4 or thioacetamide [11]. Adenosine

Electronic supplementary material The online version of this article (doi:10.1007/s11302-013-9368-1) contains supplementary material, which is available to authorized users.

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$A_{2A}R$ ligation directly stimulates collagen production by stimulated dermal fibroblasts [13, 14] and stellate cells [15].

The $A_{2A}R$ belongs to the G-protein-coupled receptor (GPCR) superfamily [16, 17] and signals via G_s signal transduction proteins which activate adenylate cyclase, increasing cytosolic cAMP [18]. Elevated intracellular cAMP activates both protein kinase A (PKA) and Epac [19–21]. Because the $A_{2A}R$ is the only G_s-coupled adenosine receptor subtype which has not been reported to also couple to the G_q protein [22, 23], it is likely that $A_{2A}R$-mediated increases in collagen expression are related to either PKA or Epac activation by cAMP. However, a prior study suggests that both PKA and Epac activation diminish collagen1 (Col1) and collagen3 (Col3) expression [24]. In support of the hypothesis that cAMP diminishes collagen production, the profibrogenic agonist transforming growth factor β (TGFβ) decreased Epac1, but not Epac2, expression in fibroblasts from multiple tissues and stimulated Col1 and Col3 expression; activation of both Epac1 and PKA inhibited Col1 and Col3 expression [25]. Moreover, targeting an increase in cAMP has been proposed as an antifibrotic strategy [24]. Since the adenosine $A_{2A}R$ is a G_s-coupled receptor that stimulates an increase in cAMP in nearly every cell tested, the mechanism by which $A_{2A}R$ regulates collagen production remains a paradox.

There is a marked decrease in the ratio of Col1 to Col3 in the matrix of scars or in the fibrous matrix of cirrhotic liver as compared to normal tissues, and blockade of adenosine $A_{2A}R$ appears to affect Col3 more than Col1 expression in scars [10]. In scars, matrix composition dictates both the strength and the appearance of the resulting scar so it is important to understand how agents that may alter the wound healing or scarring process regulate the composition of the wound matrix. Interestingly, the intracellular signaling pathways by which adenosine $A_{2A}R$s signal for expression of Col1 and Col3 differ in hepatic stellate cells; $A_{2A}R$ stimulation promotes an increase in Col1 expression via the PKA/ERK pathway but increases Col3 expression via a pathway involving activation of p38MAPK in the LX-2 hepatic stellate cell line [15]. We therefore asked how activation of $A_{2A}R$ promotes an increase of both Col1 and Col3 favoring dermal fibrosis and scarring. Here we report that $A_{2A}R$s differentially regulate Col1 and Col3 expression via a complex mechanism dependent on cAMP but proceeding via either PKA- or Epac-dependent signaling pathways.

Results

The dose–response relationship for $A_{2A}R$-stimulated increases in Col1 and Col3 differ

To better define the effects of $A_{2A}R$-mediated stimulation on synthesis of wound matrix by dermal fibroblasts, we examined the dose–response relationship for the selective $A_{2A}R$ agonist CGS21680-mediated stimulation of collagen1 (Col1) and collagen3 (Col3) by normal human dermal fibroblasts (NHDF). Since the well-known profibrotic peptide TGF-β1 increased both Col1 and Col3 after 24 h (Supplemental Figure 1), NHDF were also incubated with CGS21680 for 24 h. We were surprised to find that the concentrations of CGS21680 required to stimulate increased production of Col1 (both components: 1α1 and 1α2) and Col3 differed markedly, even though stimulation was mediated by the A_{2A} receptor since SCH58261, a highly selective $A_{2A}R$ antagonist, reverses the effect of CGS21680 on both collagens (Fig. 1). Moreover, when we analyzed the ratio of Col1:Col3 expression in NHDF stimulated with CGS21680, we found that the ratio of Col1:Col3 production decreased significantly from 4.2±0.5 to 2.9±0.3 as the concentration of CGS21680 increased from 0.1 to 10 μM. The change in Col1:Col3 ratio at differing $A_{2A}R$ agonist concentrations is likely to be physiologically and pharmacologically important since, in normal skin, where adenosine concentration varies from 30 to 300 nM, there is a Col1 to Col3 ratio of 4:1, but in hypertrophic and immature scars, where adenosine concentration is at micromolar ranges, the ratio decreases to 2:1 [4]. That $A_{2A}R$ activation increases Col1 and Col3 expression was further corroborated by quantitation of mRNA for these proteins (Fig. 1c); these results were in agreement with previously published results obtained in studies of hepatic stellate cells and the human hepatic stellate cell line LX-2 [15].

PKA activates Col1, but inhibits Col3 production after $A_{2A}R$ activation

Adenosine receptors are all members of the large family of G-protein-coupled receptors, and the $A_{2A}R$ signals almost exclusively via $G_{\alpha S}$ signal transduction proteins, which activate adenylate cyclase and mediate downstream signaling via cAMP, which leads to activation of two divergent pathways activated by protein kinase A (PKA) and the guanine nucleotide exchange factor Epac1/2. As expected, $A_{2A}R$ activation by the specific agonist CGS21680 increased the intracellular levels of cAMP in a dose-dependent fashion (Fig. 2a). To follow, we studied the impact of PKA inhibition on the CGS21680 activation of Col1 and Col3. The PKA inhibitor, PKI, dramatically decreased PKA activity (95.83±4.16 % inhibition, $P<0.01$ Student's t test; Fig. 2b) and completely blocked the effect of adenosine $A_{2A}R$ stimulation by CGS21680 on Col1 expression (Fig. 2c). In contrast, PKI alone enhanced the effect of CGS21680 0.1 μM on Col3 expression (170.2± 18.1 % of control, $P<0.001$ Student's t test).

Since three different PKA catalytic subunits have been discovered [26], we next silenced PKA-Cα, PKA-Cβ, and PKA-Cγ (Fig. 3a) which, interestingly, increased basal Col1

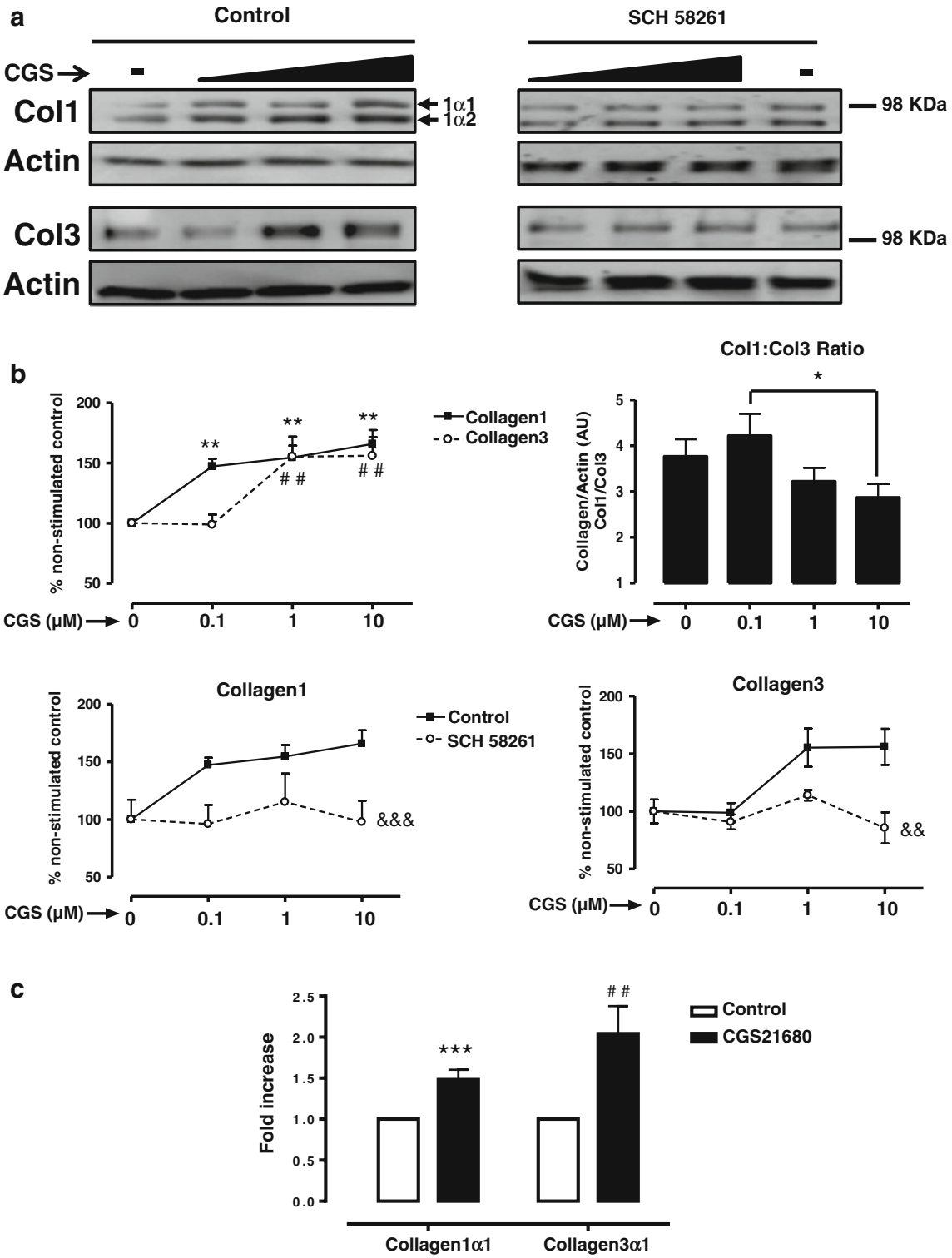

Fig. 1 $A_{2A}R$ activation increase collagen I and III with different potency. **a** NHDF cells were incubated with increasing concentrations of the $A_{2A}R$ agonist CGS21680 during 24 h, with or without pre-incubation with the $A_{2A}R$ selective antagonist SCH58261 0.1 μM. *Black triangle* represents the increasing concentrations of CGS21680 0.1–1–10 μM. **b** Densitometry of bands showing percent change and Col1:Col3 ratio. Data represent means±SEM of more than ten independent experiments, and statistics was performed by ANOVA followed by Newman–Keuls post-test, Col1 $^{**}P<0.01$ and Col3 $^{\#\#}P<0.01$ vs. non-stimulated control; $^{*}P<0.05$ CGS21680 0.1 vs. 10 μM; or by two-way ANOVA, $^{\&\&\&}P<0.001$ and $^{\&\&}P<0.01$ SCH58261 vs. control. **c** mRNA for collagen1α1 and collagen3α1 was measured by real-time RT-PCR after a 24-h stimulation with CGS21680 1 μM. Data represent means±SEM of four independent experiments, and statistical analysis was performed by Student's *t* test, collagen1α1 $^{***}P<0.001$ and collagen3α1 $^{\#\#}P<0.01$ vs. non-stimulated control

and Col3 expression. Col1 expression was unaffected by CGS21680 treatment in PKA-Cα and PKA-Cβ knockdown cells, but it was markedly increased by CGS21680 in PKA-Cγ-depleted cells. Col3 expression after $A_{2A}R$ activation was increased when compared to basal expression in all three knockdowns, although the difference was not significant in PKA-Cβ-depleted cells (Fig. 3b).

Epac2 is required for Col1 and Col3 production following $A_{2A}R$ activation

Prior studies indicate that Epac plays a significant role in regulating collagen synthesis and fibroblast migration, suggesting that Epac1 is a negative regulator of both Col1 and Col3 [25]. Therefore, we also investigated the impact of Epac on Col1 and Col3 production after $A_{2A}R$ activation by CGS21680, which robustly increased Epac activity (Fig. 4a). Next, we silenced Epac1 or Epac2 (Fig. 4b). Interestingly, Epac1 silencing increased basal expression of both Col1 and Col3 (Col1 142.2±19 %, $P<0.05$ vs. negative control; Col3 150.4±10 %, $P<0.001$ vs. negative control), and levels were also elevated upon increasing concentrations of CGS21680. However, Epac2 depletion prevented the CGS21680-induced increase in both Col1 and Col3 (two-way ANOVA, $P<0.001$), and double knockdown of Epac1/2 prevented Col3 but not Col1 increase after $A_{2A}R$ activation (Fig. 4c). These results confirm previous reports in which Epac1 was found to negatively regulate Col1 and Col3 [25] and suggest that Epac2 is required for the expression of Col1 and Col3 following adenosine $A_{2A}R$ stimulation.

Discussion

Collagen is the principal building block of connective tissue, and its upregulation in malignancies such as scleroderma is a critical event in the development of tissue fibrosis [27]. In normal skin, types I and III collagen exist in a ratio approximately 4:1, whereas in hypertrophic and immature scars, the percentage of type III collagen may be as high as 33 %, altering the ratio of Col1 to Col3 to as low as 2:1 [4]. Adenosine is present in most biological fluids and is elevated during tissue stress when it acts as a potent endogenous modulator of inflammation and tissue repair [2, 3] so that physiological interstitial levels of adenosine of 30 to 300 nM are found in normal tissues, while adenosine concentrations can reach micromolar levels during hypoxia, ischemia, inflammation, and other types of injury [28]. We therefore sought to analyze the impact of the $A_{2A}R$ agonist CGS21680 ranging from nanomolar to micromolar concentrations. It has previously been reported that adenosine, acting via the $A_{2A}R$, promotes an increase in collagen in wounds [6, 7] and in vitro [12, 14]. In fact, we have previously shown that $A_{2A}R$ stimulation promotes dermal fibrosis, as both $A_{2A}R$ antagonism and knockdown protect mice from developing bleomycin-induced dermal fibrosis and $A_{2A}R$ antagonism prevents excessive scarring by hampering Col3 overproduction compared to Col1 [10] and protects from dermal fibrosis in a model of elevated tissue adenosine [29]. We therefore hypothesized that Col1 production is more sensitive to $A_{2A}R$ activation than Col3 so that increasing concentrations of CGS21680 would decrease the Col1:Col3 ratio. Interestingly, hypoxia increases adenosine extracellular levels by suppressing both adenosine uptake and metabolism [30–33] and, at the same time, conditions of hypoxia promote fibrogenesis [34]. Moreover, HIF-1α induces $A_{2B}R$ expression [35] indicating that the $A_{2B}R$ exerts a tissue protective function [36, 37], but the $A_{2A}R$ may also contribute to the functions of adenosine in ischemic settings [35, 36]. Therefore, further studies will be needed to fully understand the interplay between hypoxia, HIF-1α, and adenosine in skin fibrosis. In this regard, and highlighting the hypoxia–inflammation relationship [30], it has been recently shown that hypoxia elicits a potent anti-inflammatory mechanism to limit tissue damage in conditions of reduced oxygen availability [38].

Among the most well-known effects of adenosine $A_{2A}R$ receptor stimulation is increasing intracellular cAMP [28, 39, 40], and $A_{2A}R$ stimulation activates both PKA [20] and Epac [19]. Moreover, the $A_{2A}R$ has been shown not to couple to the Gq/PLC/PKC pathway [22, 28]. In fact, direct activation of PKC with phorphol 12-myristate 13-acetate (PMA) dramatically inhibits both Col1 and Col3 (Supplemental Figure 2), strongly indicating that $A_{2A}R$ promotion of collagen signals via cAMP. Paradoxically, both Epac and PKA have been reported to decrease Col1 and Col3 synthesis in human fibroblasts [25], and others have suggested that stimulating increased cAMP levels or activating Epac could be used to inhibit fibrosis [24]. Thus, Yokoyama et al. showed that mRNA expression of both Col1 and Col3 is decreased by activation of both enzymes PKA and Epac using cAMP analogs that specifically activate PKA or Epac at 50 μM [25]. The work reported here clearly confirms the role of cAMP/PKA/Epac activation in adenosine $A_{2A}R$-mediated stimulation of collagen production, which is in agreement with the finding that collagen is increased by activation of other adenyl cyclase-coupled receptors such as the angiotensin AT-1R [41].

Previous reports suggest that differences in cAMP affinities between PKA isoforms contribute to specificity in the cAMP pathway [42, 43], and our results are consistent with this hypothesis. Moreover, inhibition of PKA by PKI dramatically reduced PKA activity (Fig. 2b) and prevented $A_{2A}R$-mediated stimulation of Col1 expression but potentiated Col3 expression. In agreement with previous findings,

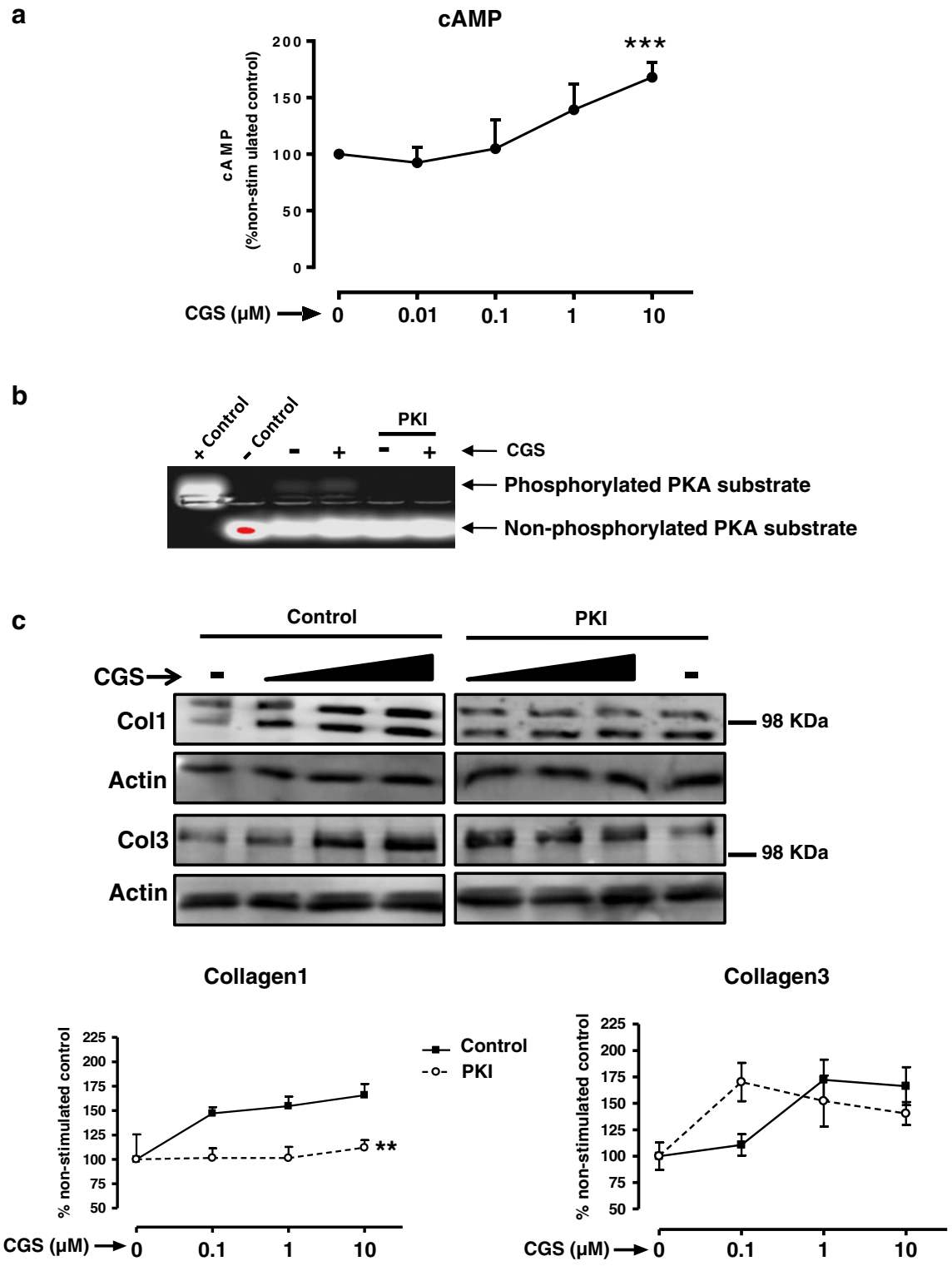

Fig. 2 PKA activates collagen I but inhibits collagen III. **a** Intracellular cAMP levels were measured as described in "Methods" section. Statistical analysis was performed by Student's *t* test, ***$P<$ 0.001 vs. non-stimulated. **b** PKA activity measurement; when indicated, 1 h prior to CGS21680 stimulation, NHDF were pre-incubated with the PKA inhibitor PKI (10 μg/ml), and PKA activity was analyzed as described in "Methods" section. **c** Cells were pre-incubated with the PKA inhibitor PKI (10 μg/ml) before CGS21680 addition and Col1 and Col3 expression analysis. *Black triangle* represents the increasing concentrations of CGS21680 0.1–1–10 μM. Statistical analysis was performed by two-way ANOVA, **$P<0.01$ PKI vs. control. Data represent means±SEM of three or more independent experiments

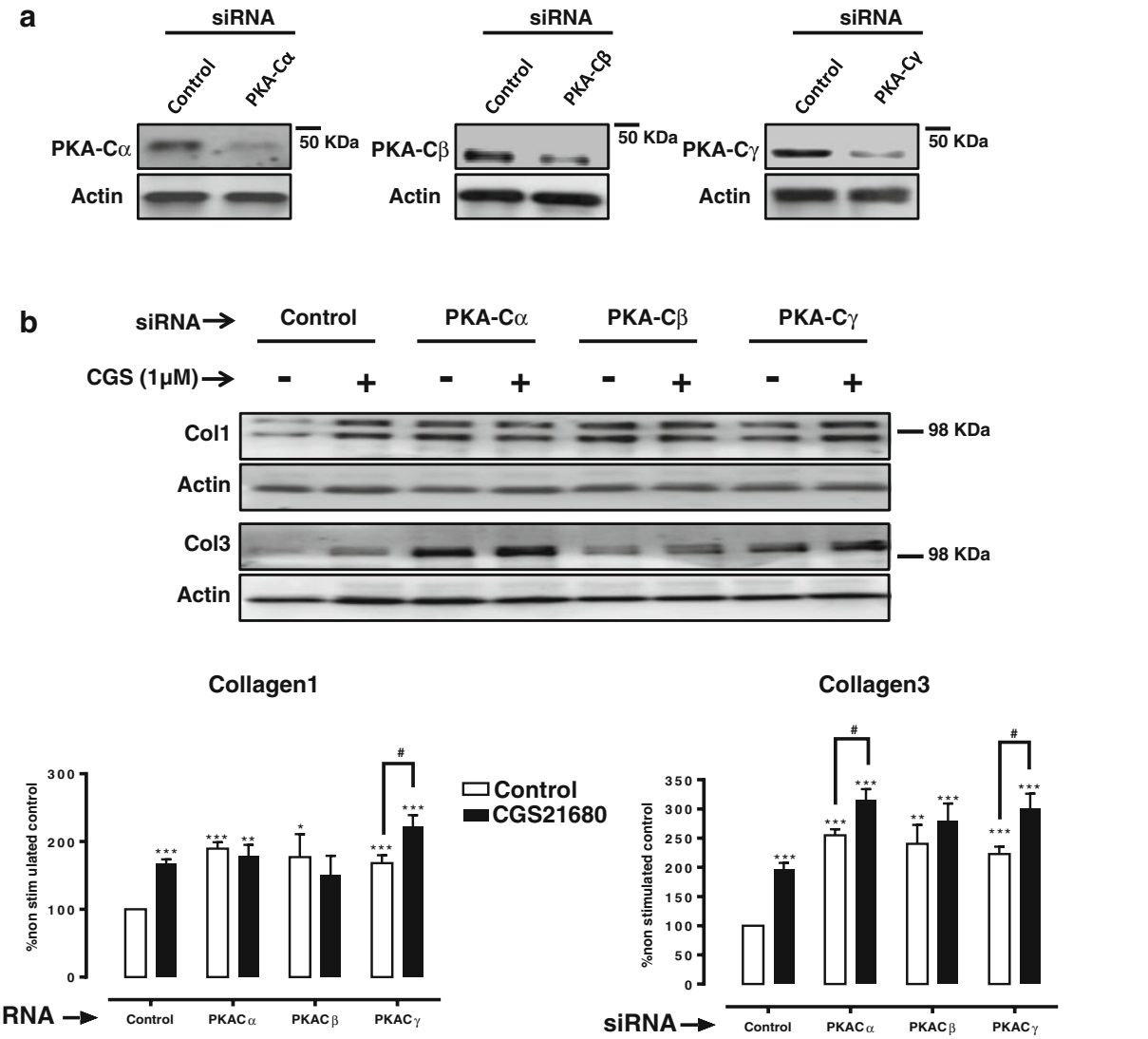

Fig. 3 Impact of knockdown of the PKA catalytic subunits on Col1 and Col3 expression. **a** siRNAs for PKA-Cα, PKA-Cβ, and PKA-Cγ selectively reduce the protein expression when compared to control siRNA. **b** Impact of PKA-Cα, PKA-Cβ, and PKA-Cγ silencing on basal and CGS21680 1-μM-increased Col1 and Col3 expression. Statistics was performed by Student's *t* test, $*P<0.05$, $**P<0.01$, and $***P<0.001$ vs. non-stimulated control siRNA and $^{\#}P<0.01$ CGS216280 vs. non-stimulated of the same siRNA. Data represent means±SEM of three or more independent experiments

knockdown of the PKA catalytic subunits increased basal Col1 and Col3 expression (Fig. 3b) [25]. To our knowledge, the present work is the first description of a differential regulation of Col1 and Col3 by PKA following $A_{2A}R$ activation: we found that the increase in Col1 following CGS21680 incubation was prevented in PKA-Cα and -Cβ-depleted cells and that, similar to the impact of the PKI inhibitor, Col3 was further increased by $A_{2A}R$ by knockdown of the catalytic subunits of PKA. These results are most consistent with the hypothesis that activation of PKA after $A_{2A}R$ activation stimulates Col1 expression but inhibits Col3 expression.

Although PKA was initially thought to be the exclusive mediator of cAMP action [44], it was subsequently recognized that Epac mediates many of the effects of cAMP on cellular function [45]. To determine whether Epac signaling mediates collagen expression in response to adenosine A_{2A} stimulation, we knocked down both Epac1 and 2 (Fig. 4b) and found that Epac1, but not Epac2, silencing increased basal Col1 and Col3 expression (Fig. 4c), which is consistent with the observation that TGF-β1 promotes collagen synthesis by repressing Epac1 but not Epac2 in fibroblasts from different tissues [25]. Interestingly, the effect of $A_{2A}R$ stimulation on both Col1 and Col3 expression was lost in Epac2-silenced cells. These findings suggest that after $A_{2A}R$ activation, Epac2 is necessary for Col1 and Col3 expression, and its regulation by G_s linked receptors.

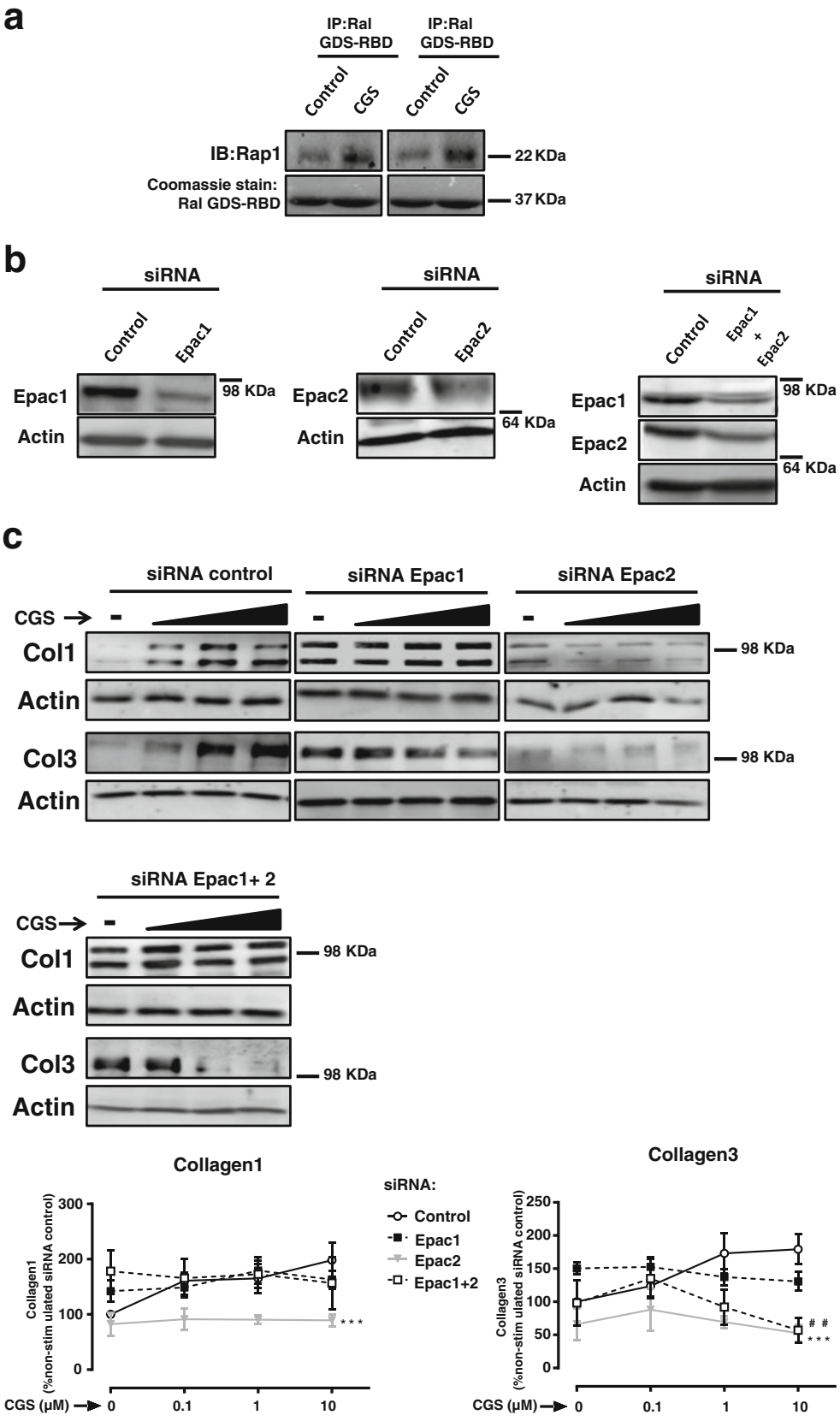

Fig. 4 Impact of Epac1/2 knockdown on Col1 and Col3 expression. **a** Epac activity was analyzed after incubation with CGS21680 1 μM for 15 min. Immunoprecipitation with Ral GSD-RBD was performed followed by immunoblot with anti-Rap1, and Coomassie stain identified Ral GSD-RBD at the predicted molecular weight of 37 kDa. **b** siRNAs for Epac1, Epac2, and double knockdown Epac1+2 selectively reduce the protein expression when compared to control siRNA. **c** Impact of Epac1, Epac2 silencing, or double knockdown Epac1+2 on basal and CGS21680-increased Col1 and Col3 expression. Statistics was performed by the two-way ANOVA: Epac2 siRNA ***$P<0.001$ and Epac1+2 siRNA $^{\#\#}P<0.01$ vs. control siRNA. *Black triangle* represents the increasing concentrations of CGS21680 0.1–1–10 μM, and data represent means±SEM of three or more independent experiments

The results of the studies presented here further highlight the dependence of $A_{2A}R$ signaling on activation of adenylate cyclase and the role of cAMP in $A_{2A}R$ signaling for fibroblast collagen production, suggesting that submaximal activation of

the $A_{2A}R$ selectively activates PKA, favoring Col1 production but repression of Col3. However, stronger activation of $A_{2A}R$ activates Epac2 to stimulate both Col1 and Col3 expression. In accord with this hypothesis, it has previously been described that although cAMP affinities for Epac and PKA are similar, cAMP binds cooperatively to PKA but not to Epac, and thereby increases the responsiveness of PKA to a slight change of cAMP in the intact cell [46]. Similarly, it has been previously described that a slight intracellular cAMP increase activates cardiac fibroblast migration, which is, nonetheless, inhibited upon micromolar cAMP [25]. By highlighting the importance of intracellular cAMP levels on collagen production, our results shed some light into how a $G_{\alpha S}$-coupled receptor, the $A_{2A}R$, increases both Col1 and Col3.

In summary, our work indicates that in NHDFs, adenosine mediates complex regulation of the Col1:Col3 balance, a determinant of the collagen quality in wounded versus normal skin, by fine-tuning intracellular cAMP levels. In Table 1, we have summarized our results showing that at nanomolar concentrations of the $A_{2A}R$ agonist (CGS_{low}), PKA activates Col1 but represses Col3, since the latter is only increased by CGS_{low} in the presence of PKI. In agreement, upon PKA C-α/C-β knockdown, basal Col1 and Col3 are increased, and CGS21680 further increases Col3 but not Col1. Similarly, Epac1 depletion increases Col1 and Col3, but Epac2 is needed at nanomolar and micromolar (CGS_{high}) concentrations for Col1 expression and at micromolar for Col3 expression. Taken together, our results suggests that at nanomolar concentrations of the $A_{2A}R$ agonist, the PKA-mediated induction of collagen I and repression of collagen III expression increases the Col1:Col3 ratio but at higher concentrations of the $A_{2A}R$ agonist, and subsequent higher intracellular cAMP concentrations, the PKA inhibition of Col3 is overcome by Epac2 activation. Moreover, the observation that there is differential regulation of Col1 and Col3 by $A_{2A}R$ provides an attractive explanation for the observation that in normal skin, where adenosine concentration varies from 30 to 300 nM, there is a Col1 to Col3 ratio of 4:1, but in hypertrophic and immature scars, where adenosine concentration is likely to be present at higher concentrations, the ratio decreases to 2:1 [4], a decrease prevented by $A_{2A}R$ blockade [10].

Table 1 Summary of the results: roles of PKA and Epac on Col1 and Col3 regulation after $A_{2A}R$ activation

Treatment	Col1	Col3	Pathway
CGS_{low}	↑	≈	$A_{2A}R$
CGS_{high}	↑	↑	
PKI+CGS_{low}	≈	↑	PKA
PKI+CGS_{high}	≈	↑	
PKA C-α/-β siRNA+CGS	≈	↑	
Epac1/2 siRNA+CGS_{low}	≈	≈	Epac
Epac1/2 siRNA+CGS_{high}	≈	≈	

The $A_{2A}R$ agonist CGS21680 was tested at 0.1 μM (CGS_{low}) and 1–10 μM (CGS_{high}) concentrations, which promoted an increase (↑), a decrease (↓), or did not affect (≈) collagen production

Methods

Antibodies, reagents, and cell line

CGS21680 and SCH58261 were purchased from Tocris Bioscience (Ellisville, MO, USA). The PKA inhibitor (TTYADFIASGRTGRRNAIHD; PKI) was purchased from Promega (Madison, WI, USA). Purified collagen1 and collagen3 and antibodies to collagen1 and collagen3 were purchased from SouthernBiotech (Birmingham, AL, USA). The RIPA buffer, protease inhibitor cocktail, the phosphatase inhibitor cocktail, phorbol 12-myristate 13-acetate (PMA), and the antibody to actin (H-196) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies and Epac1 (sc-25632), PKA-Cβ, and PKA-Cγ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Epac2 and PKA-Cα antibodies were from Cell Signaling Technology (Boston, MA, USA). TGF-β1 was from R&D systems (Minneapolis, MN, USA). NHDFs was purchased from Lonza (Walkersville, MD, USA).

Stimulation and preparation of cellular extracts and Western blot

Passage 1 to 5 of 75 % confluent NHDF cells was stimulated during 24 h with the $A_{2A}R$ agonist CGS21680 at 0.1, 1, or 10 μM. When indicated, the $A_{2A}R$ antagonist SCH58261 (0.1 μM) was added 15 min or the inhibitor of PKA (PKI; 10 μg/ml) 1 h before CGS21680. For all the experiments with inhibitors/antagonists, a dose–response from 0.1 to 10 μM of CGS21680 was run in parallel. After stimulation, cells were washed with cold PBS and lysed RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail. The protein content was measured by the BCA protein assay (Thermo Fisher Scientific, Pittsburgh, PA, USA). Three micrograms of protein extract for Col1, PKA-Cα, PKA-Cβ, and PKA-Cγ and 10 μg for Col3, Epac1, and Epac2 were separated on SDS-polyacrylamide gels. Then, proteins were transferred to PVDF membranes for immunoblotting. Prior to antibody incubation, membranes were blocked in Tris-buffered saline with 0.1 % Tween 20 (TBST) plus 3 % BSA (albumin from bovine

serum, Sigma-Aldrich). Primary antibodies were incubated overnight at 4 °C at 1/500 dilution for Col1, Col3, Epac1, PKA-Cβ, and PKA-Cγ while at 1/1,000 for Epac2, PKA-Cα, and actin. Membranes were then washed with TBST, incubated with an anti-goat IgG alkaline phosphatase-conjugated secondary antibody at 1/3,000, anti-Mouse IgG alkaline phosphatase-conjugated 1/3,000, or anti-rabbit IgG alkaline phosphatase-conjugated 1/2,000 for 1 h at room temperature, and detection was performed using the ECF substrate for Western Blotting (GE Healthcare, London). Images were captured by the Typhoon Trio (GE Healthcare). Data was analyzed and quantified with Scion Image software (Scion Corporation, Frederick, MD, USA). Band quantification was first normalized to actin and then percentage was calculated to the non-stimulated control blotted in the same membrane. For collagen1, the upper band corresponding to collagen1α1 [47, 48] is represented, and calculations for collagen1α2 provided nearly identical results. Prior to Col1:Col3 ratio calculation, standard curves with purified collagen1 and collagen3 and a loading curve with cellular extracts for both types of collagen were analyzed showing that Col1 is more abundant than Col3 in NHDF (Supplemental Figure 3).

Quantitative RT-PCR

NHDF were serum starved for 24 h and stimulated with CGS21680 1 μM for 24 h. Total RNA was extracted and purified using the RNeasy Mini Kit (QIAGEN, 74704, Valencia, CA, USA) according to the manufacturer's protocol. Relative quantification of gene expression was performed using real-time RT-PCR on Mx3005P Real-Time PCR System (Strategene, Agilent Technologies, Santa Clara, CA, USA) with SYBR Green (Agilent Technologies, 600548, Santa Clara, CA, USA) according to the manufacturer's protocol. The following primers were used in real-time PCR amplification: ACTIN forward: 5-TCACCCACACTGTGCCCATC TACGA-3, reverse: 5-CAGCGGAACCGCTCATTGCCAAT GG-3; Collagen1α1 forward: 5′-TGTTCAGCTTTGTGGAC CTCCG-3′, reverse: 5′-CCGTTCTGTACGCAGGTGATTG-3′; Collagen3α1 forward: 5′-GAAGATGTCCTTGATGT GC-3′, reverse: 5′-AGCCTTGCGTGTTCGATAT-3′. mRNA abundance was determined relative to that of ACTIN.

cAMP measurement

Intracellular cAMP was measured with the Amersham (Arlington Heights, IL, USA) cAMP Biotrak Enzyme immunoassay system. Briefly, 75 % confluent NHDF were starved for 24 h and incubated at the indicating concentrations of CGS21680 for 20 min. cAMP levels were analyzed by using the non-acetylation EIA procedure according to the manufacturer's protocol.

Protein kinase A assay

The non-radioactive PepTag PKA assay (Promega, Madison, WI, USA) was used to measure PKA activity from cell lysates. The PepTag PKA assay is based on the phosphorylation of the fluorescent PKA substrate peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) (PepTag A1 peptide) which, upon phosphorylation by PKA, acquires a negative charge and can be separated from the non-phosphorylated peptide by agarose gel electrophoresis. Ninety percent confluent NHDF in 100-mm culture wells were serum starved for 24 h and stimulated for 15 min with CGS21680 (1 μM). When indicated, the PKA inhibitor PKI (10 μg/ml) was added 1 h before CGS21680. Positive and negative controls and spectrophotometric quantification of kinase activity following agarose solubilization were performed according to the manufacturer's protocol.

Rap1 activation assay

Ninety percent confluent NHDF in 100-mm culture wells were serum starved for 24 h and stimulated for 15 min with CGS21680 (1 μM). Rap1 activation assays using a GST-tagged fusion protein corresponding to amino acids 788–884 of the human Ral-GDS-Rap binding domain bound to glutathione agarose (Ral GDS-RBD agarose) were performed using a Rap1 activation assay kit according to the manufacturer's directions (Millipore, Billerica, MA, USA).

RNA interference (siRNA)

Double-stranded siRNAs to Epac1 (s20360), Epac2 (s21816), the catalytic subunits PKA-Cα (s11065), PKA-Cβ (s11068), and PKA-Cγ (s11071) and negative siRNA (4390844) used as a control were purchased from Ambion (Life Technologies, Grand Island, NY, USA). Cells were transfected with siRNA (5 pmol for Epac1; 50 pmol for Epac2, PKAα, PKAβ, and PKAγ; and 25 pmol of each for the double knockdown, with the negative control at the respective concentrations), using Lipofectamine RNAiMAX (Invitrogen, Life Technologies, Grand Island, NY, USA). After 24 h, CGS21680 was added for 24 h, and cellular extracts were prepared as described above.

Statistical analysis

Statistical differences were determined using two-way ANOVA, repeated measures ANOVA followed by Newman–Keuls post-test, or Student's *t* test carried out using GraphPad software on a PC. The alpha nominal level was set at 0.05 in all cases. A P value of <0.05 was considered significant.

Acknowledgements This work was supported by grants from the National Institutes of Health (AR56672 and AR54897), NYU-HHC (Health and Hospitals Corporation) Clinical and Translational Science Institute (UL1RR029893), NYU Cancer Institute Center Support Grant, and NIH/National Cancer Institute (5 P30CA16087-31).

Conflicts of interest AM and BNC have filed a patent on use of adenosine A2AR agonists to prevent prosthesis loosening (pending). MP-A does not have any disclosures. BNC holds patents numbers 5,932,558; 6,020,321; 6,555,545; 7,795,427; adenosine A1R and A2BR antagonists to treat fatty liver (pending); and adenosine A2AR agonists to prevent prosthesis loosening (pending). BNC is a consultant for Bristol-Myers Squibb, Novartis, CanFite Biopharmaceuticals, Cypress Laboratories, Regeneron (Westat, DSMB), Endocyte, Protalex, Allos, Inc., Savient, Gismo Therapeutics, Antares Pharmaceutical, Medivector, King Pharmaceutical, Celizome, Tap Pharmaceuticals, Prometheus Laboratories, Sepracor, Amgen, Combinatorx, Kyowa Hakka, Hoffman-LaRoche, and Avidimer Therapeutics. BNC has stock in CanFite Biopharmaceuticals.

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