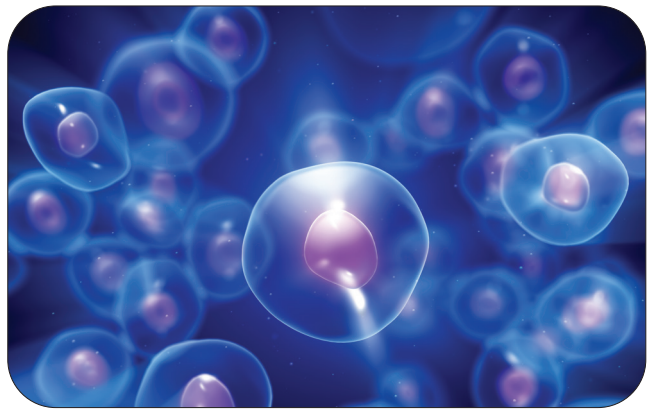
LITERATURE REVIEW

Investigating GPCR contribution to anti- and pro-fibrotic signaling with HTRF, AlphaScreen, and DELFIA assays

Abstract

Fibrotic disorders are a collection of complex pathologies that are thought to result from improperly monitored and excessive wound healing mechanisms. The pathogenesis of these disorders is not well understood and some GPCR signaling pathways may be involved in the processes that regulate the cell types responsible for wound healing and therefore fibrosis. This note gathers publications that exemplify various applications of HTRF™, AlphaScreen™ and DELFIA™ assays to investigate the role of GPCR signaling in fibrosis development.



Introduction

G-protein coupled receptors (GPCRs) are a large family of transmembrane receptors that are involved in almost every metabolic mechanism. There are about 800 GPCRs in humans and they are the most important area of therapeutic research and the most represented target family in FDA-approved drugs. They signal inside cells using a variety of mediators including G-proteins, β-arrestins and other ligands, to fulfill many roles in cell regulation across all organs. As such, they have become an area of research in fibrosis as in many other diseases.

revvity

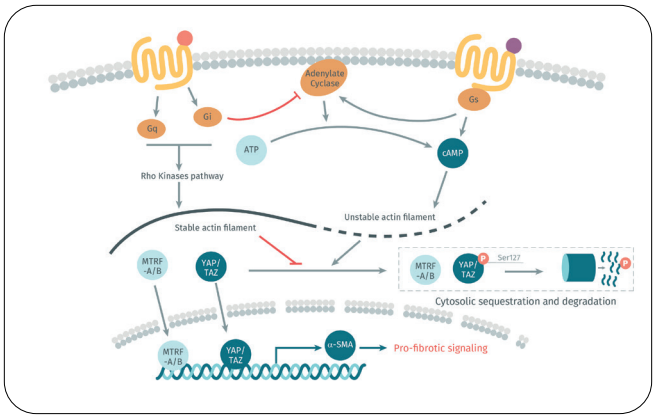


Figure 1: Example of GPCR-driven regulation of pro- and anti-fibrotic processes in lungs. On the one hand, Gs proteins promote adenylate cyclase activity and increases in cAMP levels, which results in PKA activation and unstable actin filaments. On the other hand, Gi proteins have an opposite action and -along with Gq proteins- promote the Rho kinase pathway, which results in stable actin filaments. Actin filaments' stable or unstable status regulates the cytosolic sequestration and degradation of mediators MTRF-A/B and YAP/TAZ, which promotes proliferation gene expression including the pro-fibrotic mediator α-SMA.

In fibrosis, the unregulated differentiation of wound healing fibroblasts into myofibroblasts is assumed to be a critical step on the development of fibrotic disorders. In this context, GPCRs are especially investigated under the hypothesis that some of them may have profibrotic or antifibrotic effects by regulating the differentiation of fibroblasts into myofibroblasts (see Figure 1). The diverse and dynamic expression of GPCRs in cells adds a layer of complexity to the understanding of their roles in fibrosis.

Some key GPCR ligands and their corresponding receptors have already been identified as potential players in the development of fibrotic disorders and are being investigated regarding various organ fibrosis. For example, on the one hand, angiotensin and endothelin signaling through GPCRs may induce fibroblast differentiation and therefore participate to the pathogenesis of pulmonary fibrosis. On the other hand, relaxin is suggested to promote protection against such disorders in the heart and kidneys.

GPCR-Dependent camp levels in lung fibroblast proliferation

Roberts, Metal. Respiratory Research (2018) 19:56

Idiopathic pulmonary fibrosis is a chronic fibrotic disease of the lung with poor outcomes, which is believed to arise from abnormal wound healing following repetitive injuries and inflammation. Recent evidence suggests the activation of pro-cAMP mechanisms may result in a downregulation of pro-fibrotic pathways via an inhibition of the wound-healing fibroblasts differentiation and proliferation.

In 2018, Roberts, et al. investigated the human lung fibroblast GPCR repertoire to identify which GPRC families could be studied for best cAMP increase and inhibition of fibroblast differentiation and proliferation. Using different ligands to target and stimulate different families of GPCRs, they monitored the proliferation of human lung fibroblasts using Revvity's DELFIA proliferation assay (Figure 2), which monitors the incorporation of BrDU in place of thymidine in newly synthesized DNA

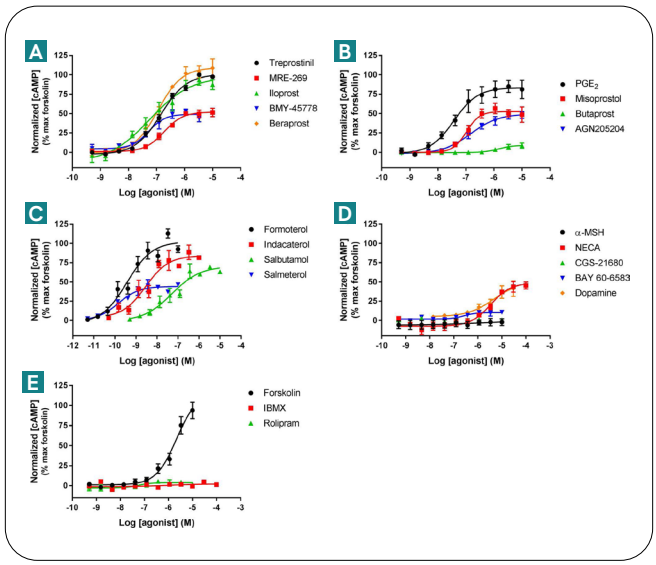


Figure 2: Receptor-mediated cAMP accumulations in human lung fibroblasts.Concentration effect curves for cAMP accumulation in HLF after treatment with a range of agonists targeting (a) IP receptors, (b) EP receptors, (c) b2 adrenoceptor, (d) other GPCRs, (e) non-receptors. Taken from: Roberts, et al.

In that assay cells are incubated with the non-radioactive pyrimidine analog BrDU. Upon cell proliferation, BrDU competes with thymidine and is incorporated in newly synthesized DNA. The amount of BrDU incorporated is therefore proportional to cell proliferation. Europium labelled anti-BrDU antibodies are then used to measure the level of BrDU incorporation via time-resolved fluorescence.

cAMP levels in human lung fibroblasts was monitored in similar conditions using Revvity's AlphaScreen cAMP assay (Figure 3). Normalization of cAMP levels across populations was achieved using the adenylate cyclase activator, forskolin.

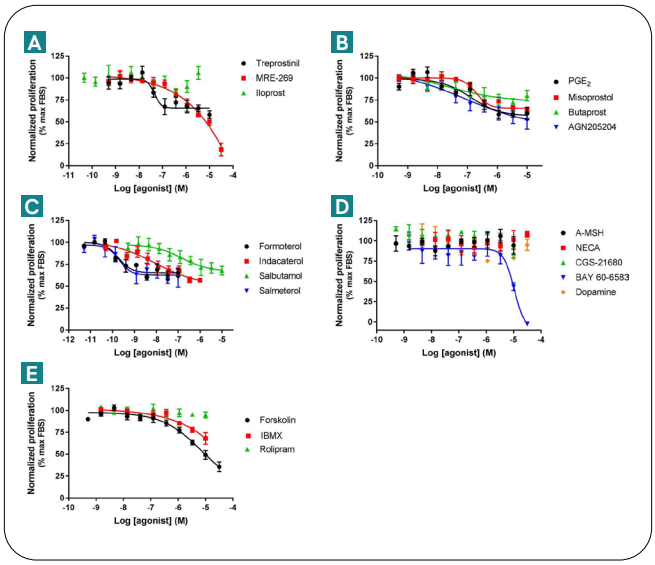


Figure 3: Receptor-mediated of serum-mediated proliferation in human lung fibroblasts. Concentration effect curves for the inhibition of proliferation were determined in HLF after treatment with a range of agonists targeting) (a) IP receptors, (b) EP receptors, (c) b2 adrenoceptor, (d) other GPCRs, (e) non-receptors. Taken from Roberts et al.

Results from both cAMP measurement and proliferation assays were then computed in a correlation plot to identify potential links between the two (Figure 4).

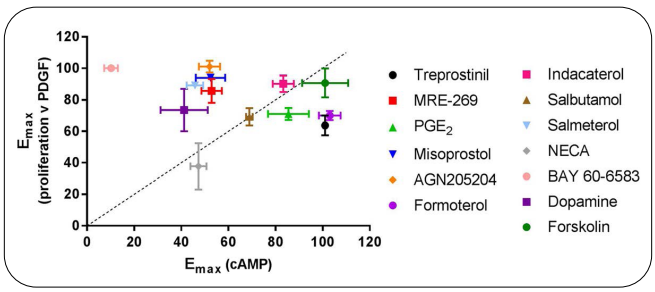


Figure 4: Pearson correlation plot between global cAMP accumulation and inhibition of PDGF-mediated proliferation of human lung fibroblasts for several GPCR agonists. Taken from Roberts et al.

The study was successful at identifying some of the receptors tested (IP, β2, EP2, EP4, and A2B) as having signaling abilities that inhibits pro-fibrotic hallmarks like the differentiation and proliferation of fibroblasts.

However, the results show that acute cAMP accumulation did not correlate with those anti-fibrotic outcomes. The authors identified that once a certain level of cAMP was reached, further accumulation did not yield better results in terms of fibroblast differentiation and proliferation.They suggest acute cAMP increases are therefore not a relevant parameter in predicting anti-fibrotic outcomes but should incorporate other factors such as the spatio-temporal behavior of cAMP levels.

At1r-At2r-Rxfp1 crosstalk in myofibroblasts activity

Chow, B,etal. JASN 30: (2019) 2191-2207

Recombinant human relaxin-2 (serelaxin) is known to mediate organ-protective signaling through its corresponding G-protein coupled receptor RXFP1. Relaxin effects are mediated via the nitric oxide pathway, which promotes both NO and cGMP, the later respectfully inhibits pro-fibrotic TGF-β1 signaling and vasodilation (Figure 5). After this organ protection was suggested to include anti-fibrotic functions, some studies confirmed relaxin-mediated improvements in kidney fibrosis and found the angiotensin II receptor (AT2R) to be essential in that mediation (Figure 6).

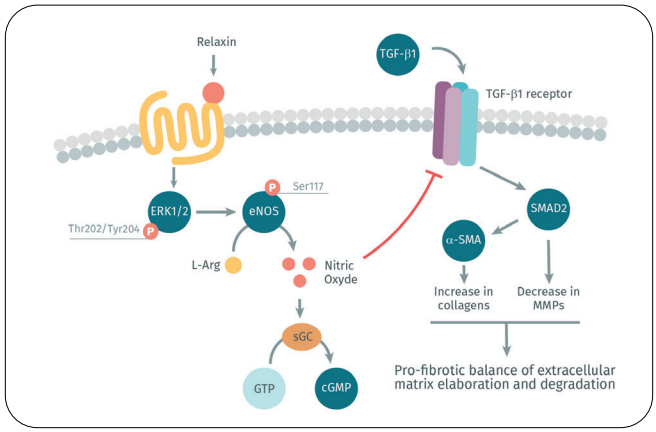


Figure 5: Relaxin signaling via RXFP1 and the nitric oxide pathway promotes the inhibition of TGF-b1 pro-fibrotic signaling pathway.

In a 2019 study, Chow, et al. sought to better understand the ties between the anti-fibrotic effects of relaxin and ATR2 as it was not known whether the two interact directly or if they exhibit other collaborative behaviors.

As a reading of relaxin signaling via the anti-fibrotic nitric oxide pathway, the authors monitored cGMP levels in rat renal myofibroblasts and human cardiac myofibroblasts expressing all RXFP1 and both angiotensin receptors AT1R and AT2R. Using Revvity's HTRF cGMP kit and inhibitors of all three studied receptors, the team measured the intensity of relaxin signaling in relation to AT1R and AT2R activity (Figure 6).

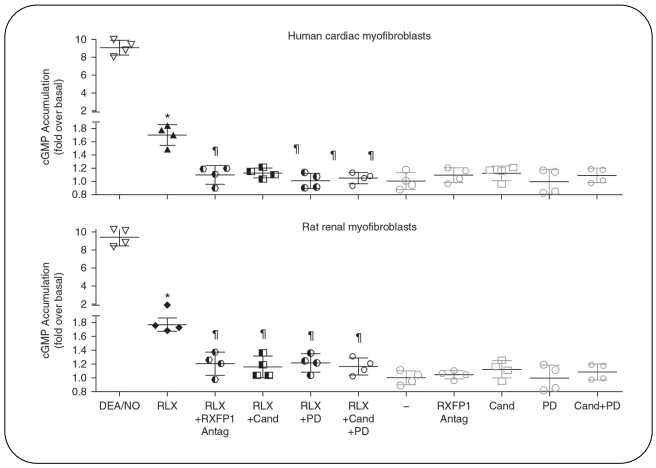


Figure 6: Evidence for acute AT1R-AT2R-RXFP1 interactions on HCMFs and RRMFs. cGMP accumulation was significantly increased by RLX (1 nM) treatment of HCMFs or RRMFs. The cGMP-promoting effects of RLX in HCMFs and RRMFs were significantly inhibited by pretreatment with an RXFP1 antagonist, AT1R antagonist (candesartan) alone, AT2R antagonist (PD123319) alone, or AT1R & AT2R antagonists combined. Each antagonist alone, however, did not significantly affect basal cGMP measurements. Copyright : Republished with permission. From Chow, B, et al. (2019) AT1R-AT2R-RXFP1 Functional Crosstalk in Myofibroblasts: Impact on the Therapeutic Targeting of Renal and Cardiac Fibrosis. JASN 30: 2191–2207

These results led the authors to support the idea of an AT1R-AT2R-RXFP1 crosstalk whose regulation plays a relevant role in pro- and anti-fibrotic pathways. They add that this axis may be open for allosteric modulation that could result in pro-fibrotic signaling but would constitute an unexplored area or therapeutic research for fibrosis.

Chronic Kidney Disease (CKD)

Akli Ayoub, M, et al. PLoS One. 2016; 10(3)

Chronic kidney disease (CKD) gathers a collection of symptoms including chronic inflammation, infiltration of inflammatory cells, cellular death and fibrosis. While the exact pathogenesis of these disorders is unknown, angiotensin II (AngII) -the natural ligand of the angiotensin family of receptors AT1R and AT2R- is understood to contribute to the initiation and progression of the disease. It was suggested by recent studies that parts of AngII pathological effects in CKD result from a functional relationship between AT1R and the chemokine receptor CCR2, but the nature of that relationship remains unclear.

In a 2015 study, Akli Ayoub & Yang, et al. aimed to explore the functional interactions between the angiotensin II (AngII) and CCL2 receptors (AT1R & CCR2). They conducted this research *in vitro* with HEK293FT cells and *in vivo*, with Sub-Total Nephrectomized (STNx) rats, a well-established CKD model. These rats are characterized by a massive renal mass ablation and present, among other symptoms, tubulointerstitial fibrosis.

Among other experiments, they used the HTRF IP-One assay to monitor Gq coupled GPCRS AT1R and CCR2 biological activity when stimulated with AngII and CCL2.The assay involved different transfected strains expressing either AT1R, CCR2 or both (Figure 7).

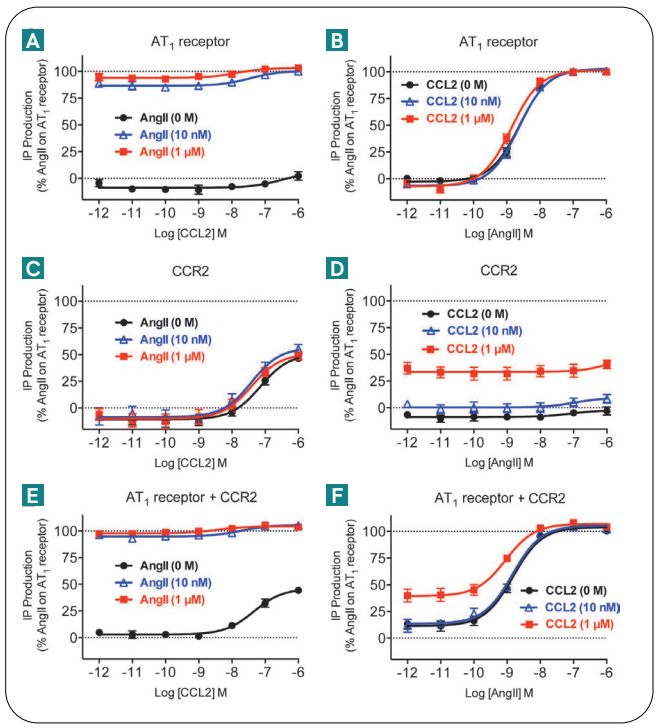


Figure 7: Biological response (IP-1 accumulation) in HEK293FT cells expressing or co-expressing AT1R and CCR2 HEK293FT cells expressing AT1R (A and B), CCR2 (C and D) or both AT1R and CCR2 (E and F) were used to measure agonist-induced IP-1 production with increasing doses of CCL2 in the presence of AngII (A, C and E) or with increasing doses of AngII in the presence CCL2 (B, D and F). Taken from Akli Ayoub & Yang, et al.

AngII stimulation of AT1R-expressing cells resulted in a biological response (measured in IP-1 accumulation) both efficient and potent (Figure 7b), while the stimulation of CCR2-expressing cells with CCL2 resulted in a response of lesser efficacy and potency (Figure 7c). The response to either AngII or CCL2 of cells co-expressing AT1R and CCR2 did not result in any apparent interactive effects (Figure 7e & f). These results led the authors to conclude the

collaboration of AT1R and CCR2 do not result from a shared ligand signaling but is rather dependent on conformational changes one of the receptors forces upon the other when activated.

Additional experiments involving BRET assays supported this hypothesis and the authors concluded on either a direct allosteric modulation of CCR2 by active AT1R or an uneven β-arrestin recruitment at both receptors with stronger G-protein signaling inhibition at CCR2.

Conclusion

The publications gathered in this note show how GPCR research contribute to fibrosis development understanding and therapeutic research in that area. It also introduces how HTRF, AlphaScreen and DELFIA assays have been a relevant tool for fibrosis related research for years and proved to be successful at handling the range of applications and experiments necessary to advance the knowledge of this area.

References

Roberts, M et al. The inhibition of human lung fibroblast proliferation and differentiation by Gs coupled receptors is not predicted by the magnitude of cAMP response. Respiratory Research (2018) 19:56

Chow, B, et al. AT1R-AT2R-RXFP1 Functional Crosstalk in Myofibroblasts: Impact on the Therapeutic Targeting of Renal and Cardiac Fibrosis. JASN 30: (2019) 2191–2207

Akli Ayoub, M, et al. Functional Interaction between Angiotensin II Receptor Type 1 and Chemokine (C-C Motif) Receptor 2 with Implications for Chronic Kidney Disease. PLoS One. 2016; 10(3)

Products used

- DELFIA™ cell proliferation assay
- AlphaScreen™ cAMP assay
- HTRF™ IP-ONE assay

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