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The role of β-arrestins in respiratory pathophysiology and tumorigenesis: going a step beyond the cell surface

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Abstract. – Beta-arrestins are small cytosolic proteins that have been known so far as negative feedback regulators of G-protein coupled receptors (GPCRs). This receptor superfamily, characterized by a heptahelical transmembrane motif, mediates the signals of a multitude of extracellular ligands including chemokines, cytokines, hormones and growth factors. Beta-arrestins "arrest" the GPCR signaling capability through its desensitization and internalization. However, novel roles for these molecules have emerged and research demonstrates that beta-arrestins can mediate intracellular signaling independently of their effects on G-protein stimulation. Acting as scaffolding proteins, they can lead to the assembly of intracellular signalsomes that can activate or inhibit the function of various signaling cascades, such as the MAP kinase, JNK and NF-kappaB cascades, ultimately affecting gene expression. Finally, they can even regulate gene transcription by modulating histone acetylation and chromatin assembly. This pleiotropic activity of beta-arrestins can regulate both physiologic and pathophysiologic responses and will be reviewed in the context of lung inflammatory diseases and lung cancer.

Key Words:
Beta-arrestins, Lung, Cancer, Asthma, COPD.

Introduction

G-protein coupled receptors (GPCRs) represent the largest and most ubiquitous family of cell surface receptors (> 1000 members in humans) and are characterized by the classical heptahelical motif that spans the cell membrane. Among them, the β_2-adrenergic receptor (β_2-AR) is present on the surface of bronchial smooth muscle cells and its activation leads to bronchodilation[1]. These receptors transduce extracellular signals into intracellular events via the activation of the G protein heterotrimeric complex. The dissociation of the G_a subunit from the $G_{\beta\gamma}$ subunit activates cell signaling systems like the adenylate cyclase, protein kinases, phospholipases and ion channels which ultimately lead to a physiological response[2]. The function of GPCRs is regulated by a group of small cytosolic proteins called arrestins that mediate their desensitization and internalization. The family of arrestins is comprised of four molecules, namely arrestins 1 and 4 that are exclusively confined to the cones and rodes of the retina and arrestins 2 and 3 (β-arrestin 1 and 2 respectively) that are present diffusely in all mammalian tissues[3]. Both β-arrestin 1 and β-arrestin 2 are responsible for the desensitization and internalization of the GPCRs through four discernible steps that involve: (1) The binding of the respective agonist ligand and the subsequent activation of the receptor; (2) the activated receptor going through a conformational change that is transmitted to its carboxyl (cytosolic) terminus tail; (3) its phosphorylation by a specialized kinase that belongs to the family of G protein-coupled receptor kinases (GPKs, GPK2 for the β_2-AR) that can specifically phosphorylate serine and threonine residues of the cytosolic tail of the agonist-occupied receptor; and (4) the binding of the β-arrestin molecules which is responsible for the desensitization and clathrin-mediated internalization of the receptor[4]. The desensitization step renders the receptor unable to mediate signals to the heterotrimeric G protein complex and, thus, terminates G protein activation. The internalization of the receptor to clathrin-coated pits can lead to either the recirculation of the receptor back to the cell membrane via acidified vesicles or its degradation through trafficking to lysosomes. Receptors have been classified into two groups depending on their rate of recycling, with type A receptors such as the β_2-AR recycling more

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rapidly. They are de-phosphorylated, resensitized and returned to the cell surface in about 30 minutes. By contrast, type B receptors recycle much more slowly and most of the receptors remain in endosomes, even after an hour, and are still associated with β-arrestins[5].

Apart from this classical role exhibited by β-arrestins, more recent research has unveiled new roles for these molecules. Acting as scaffolding proteins, they can regulate signaling pathways, particularly **M**itogen-**A**ctivated-**P**rotein-**K**inase (MAPK)/**E**xogenously-**R**egulated-**K**inase (ERK) and **N**uclear **F**actor-κB (NF-κB) pathways that are involved in pro-inflammatory gene transcription, cell differentiation, proliferation, and apoptosis[6-8]. The internalization step that has long been thought solely as an inhibitory feedback mechanism has now emerged as a means of mediating signals from the receptor to the nucleus via a complex array of protein-protein interactions and protein kinase pathways, leading to gene transcription regulation.

The Role of β-arrestins as Desensitization and Internalization Mediators of the β_2-adrenergic Receptor

B_2-adrenergic agonists represent one of the mainstays of treatment for those suffering from asthma and chronic obstructive pulmonary disease (COPD)[9-11]. Their continued use has raised concerns about the development of tachyphylaxis to their therapeutic effect backed up by evidence showing asthmatics not sufficiently managed by beta-agonist therapy, while continuous use of inhaled beta-agonists has been shown to result in a loss of their bronchoprotective effect and deterioration of asthma control[12,13]. One possible mechanism that limits their therapeutic efficiency is the desensitization of the β_2-AR, which has been shown to occur in numerous cell types after both acute and chronic exposure to beta-agonists. *In vitro* studies conducted in cell lines expressing recombinant β_2-ARs on their surface have demonstrated the role GPKs and β-arrestins play in mediating receptor desensitization[14,15]. By binding to the phosphorylated tail of the agonist-occupied β_2-ARs, β-arrestins hinder their interaction with the stimulatory G_s protein and thus "arrest" their signaling function, while their subsequent internalization downregulates the number of expressed β_2-ARs on the cell surface. The overexpression of β-arrestin 1 or β-arrestin 2 in cultured human airway smooth muscle cells (ASMs), leads to the desensitization and attenuation of beta-agonist-stimulated signaling[16]. The effects of β-arrestin 2 gene ablation were also studied in both *in vivo* and *ex vivo* murine models of ASM contractile function. In these experiments, ASM cells lacking β-arrestin 2 showed greater c-AMP accumulation after agonist stimulation[17]. The desensitization action of β-arrestins occurs within seconds to minutes after agonist-receptor binding while the internalization of the receptor represents a more delayed response[15,18]. Among the two β-arrestins, β-arrestin 2 binds more readily and to a greater extent to the β_2-AR than β-arrestin 1 although their quantitative effects on β_2-AR functional regulation has not been yet determined due to the fact that knockout of both β-arrestin genes is lethal[19,20]. Interestingly, nitric oxide (NO) which is known to be produced in greater quantities during chronic inflammatory lung diseases, such as asthma and COPD, has been shown to counteract the desensitization and internalization of β_2-AR by decreasing GPK2-mediated phosphorylation and recruitment of β-arrestins to the receptor[21].

The rate of β_2-AR desensitization and internalization could also be governed by the intrinsic efficacy of the ligand occupying the receptor. $Beta_2$-agonists represent today the mainstay of treatment for asthma and COPD[9-11]. They are classified according to the duration of their bronchodilation effect into short-acting β_2-agonists (SABA) and long-acting β_2-agonists (LABA) (Figure 1). LABA can provide bronchodilation and protection against provocative stimuli for at least 12 h after a single dose[10]. The continued use of LABA into every day practice for patients suffering from asthma and COPD has raised concerns in relation to their downregulation effect on β_2-ARs, linked to the observation that both of the asthma mortality epidemics in New Zealand and

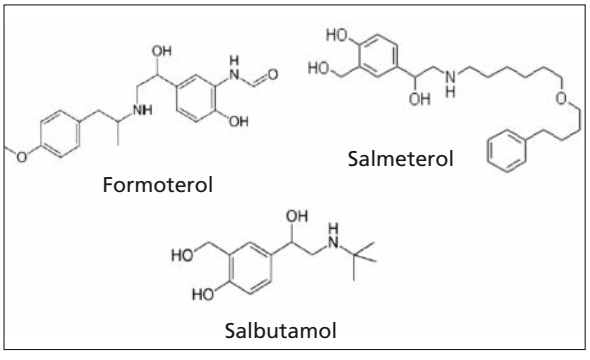

Figure 1. The LABA (Formoterol, Salmeterol) and SABA (Salbutamol) in use for COPD and asthma.

the United Kingdom were associated with the use of high-dose formulations of agonists of high intrinsic efficacy[22,23]. The intrinsic efficacy of the beta-agonist seems to determine the rate of β_2-AR phosphorylation by GPK 2, β-arrestin translocation to the cell membrane and subsequent endocytosis of the receptor. In this matter, salmeterol induced lower rates of GPK-site β_2-AR phosphorylation and internalization compared to the full agonist epinephrine and the partial agonists albuterol and formoterol[24]. Therefore, even the intrinsic properties of the beta-agonist used may determine the rate of β-arrestin recruitment and the rate of downregulation of the activated receptor.

Beta-arrestins Act as Intracellular Signaling Molecules in a G-protein Independent Manner

Asthma is a chronic inflammatory airway disease characterized by lung infiltration of eosinophils, which represent the principal effector cells, and orchestrated by T helper cells (Th_2 cells). The lung elaboration of various chemokines and cytokines is necessary for the eventual migration of the inflammatory cells to the airways[25]. In a murine model of ovalbumin-induced asthma, ablation of the β-arrestin 2 gene resulted in inhibition of inflammatory cell influx into the allergen-challenged murine airway and prevented the development of airway hyperesponsiveness (AHR)[26]. In particular, allergen-sensitized β-arrestin 2 knockout mice displayed low levels of lung eosinophils and T lymphocytes and reduced levels of Th_2 cytokines, thus preventing the development of allergic asthma, without interfering with innate immunity. The possible mechanisms mediating this effect could be due to the scaffolding function of β-arrestins, linking GPCRs to intracellular signaling pathways such as: (1) The p38 MAPK cascade, (2) the ERK 1/2 cascade and (3) The c-**J**un **N**-terminal **K**inase 3 (JNK3) cascade[8,27,28]. This supposition is supported by several *in vitro* reports which demonstrate that chemotaxis of immune and other cell types is promoted by β-arrestin-dependent activation of MAPK signaling pathways[29]. Apart from their role in regulating the function of nuclear transcription factors, the β-arrestin-2/MAPK scaffolds are linked preferentially to the phosphorylation of cytosolic substrates (like the small GTPase RhoA, described later on) which can mediate cell chemotaxis[30,31]. One other option is the desensitization and internalization action of β-arrestins on GPCRs that function as chemokine receptors, keeping in line with their more traditional role. For instance, CCR3, which represents the main chemokine receptor for eosinophils, expresses multiple carboxyl-tail serine/threonine-rich regions which can undergo agonist-induced phosphorylation and are a likely binding site for β-arrestins[32,33]. B-arrestin 2 may also control the differentiation of naïve T cells into T helper cells. A study has demonstrated that β-arrestin 2 is necessary for the T cell antigen receptor-mediated activation of the Ras-ERK/1/2 pathway, which enhances the IL-4 receptor signaling and final differentiation of the T cell into a Th_2 cell[34].

Non-hematopoietic, lung structural cells like ASMs, mast cells and epithelial cells also regulate AHR through bronchoconstriction and cytokine elaboration. $Beta_2$-ARs functionally regulate lung structural cells in the context of allergic asthma[35]. In particular, airway epithelial MAPK signaling has been found to be important in expression of the asthma phenotype in mice and MAPKs are prominently activated by β-arrestin 2[20,36]. Additionally, airway epithelial cells were shown to express significantly more β_2-AR and symbol-arrestin 2 than airway smooth muscle cells[17]. Chimeric mice that could produce β-arrestin (+/+) hematopoietic cells but had β-arrestin (-/-) lung structural cells did not develop AHR, even in the presence of airway inflammation[37]. As β-arrestins are responsible for the desensitization and endocytosis of the β_2-AR, their lack thereof prevents its tachyphylaxis and sustains the β_2-AR-dependent bronchodilation. Lung structural cells are a target for, and a source of, pro-inflammatory factors such as cytokines and chemokines that, via autocrine and paracrine actions, actively contribute to and regulate the airway inflammatory processes and perpetuate AHR[38,39]. The lack of β-arrestins negates their effective signaling through β-arrestin 2-dependent pathways and prevents, therefore, AHR development.

Another molecule that plays a significant role in allergic diseases such as asthma is the anaphylatoxin C3a, which is produced during bacterial infection and from IgE/FceRI stimulated human mast cells[40]. It has both opsonization and chemotactic properties and plays an important part in innate immunity[41]. It induces degranulation of mast cells through its binding to its receptor C3aR and subsequent phospholipase C activation, increasing the intracellular Ca^{+2} concentra-

tion[42,43]. The degranulation process appears dependent upon β-arrestin 1, while β-arrestin 2 is responsible for the internalization of the C3aR[44]. The internalized β-arrestin 2/C3aR complex is able to bind to IκBa (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) which binds and inactivates NF-κB, hindering its translocation to the cell nucleus where it normally induces the expression of cytokine genes. β-arrestins have been shown to inhibit or activate NF-κB, depending on the cell type and receptors utilized[44].

Polymorphonuclear leukocytes (PMNs) are one of the principal cells involved in COPD, through their elaboration of cytokines and proteolytic enzymes that ultimately lead to increased inflammation, tissue destruction and remodeling[45]. The PMN levels are increased in sputum of normal smokers and they increase even further in COPD, where they correlate positively with disease severity[45]. Their function seems to be inherently regulated by β-arrestin 2 as was demonstrated in studies involving β-arrestin 2 knockout mice and wild-type mice. PMN from β-arrestin 2 (-/-) mice demonstrated augmented chemotaxis, increased expression of adhesion molecules and greater production of superoxide anion, in comparison to PMN derived from wild-type mice[46]. As already pointed out, many chemotactic and pro-inflammatory signals are mediated through GPCRs and it is plausible that β-arrestins can attenuate these signals through their classical role as desensitization and internalization mediators of the GPCRs of chemokines and cytokines. The aforementioned data contradict the findings presented in the previous paragraph, concerning the chemotaxis of lymphocytes and eosinophils, which indicates that the function of β-arrestins depends upon the cell studied and the receptor activated. Through a different mechanism described later on, β-arrestins stimulate chemotaxis by active reorganization of the actin cytoskeleton and with cell migration being attenuated when β-arrestin function is abolished by small interfering (si) β-arrestin RNA. Therefore, β-arrestins modulate cell migration through different pathways and GPCRs and, depending on the cell population, they can either stimulate or abrogate chemotaxis.

Angiotensin II (AngII) has emerged as a molecule that has pro-inflammatory and chemotactic properties, apart from its classical physiological role as a vasocontrictive agent and inducer of aldosterone secretion[47,48]. Its actions are mediated via its two receptors AT_1R and AT_2R, which belong to the GPCR superfamily. Acting mainly through AT_1R, it can contribute to the recruitment of inflammatory cells into tissue through the regulation of adhesion molecules and chemokines and by directly activating chemotaxis for monocytes, T cells and neutrophils[49-51]. The possible contribution of AngII and the locally expressed renin-angiotensin-aldosterone (RAS) systems to both COPD and asthma progression has been recently reviewed by the Authors[52]. Apart from its ability to act through G-protein complex activation, AT_1R may also mediate G-protein independent, β-arrestin 2-dependent signaling cascades. An example of the latter is the Ras-Raf-MEK-ERK 1/2 MAPK cascade that results in phosphorylation of ERK 1/2, which leads to its nuclear translocation and to active gene transcription[53-57]. AngII and AT_1R stimulate the induction of **R**egulated upon **A**ctivation, **N**ormal **T**-cell **E**xpressed and **S**ecreted (RANTES) cytokine, the potent monocyte chemoattractant IL-6, **T**umor **N**ecrosis **F**actor-α (TNF-α) and **M**acrophage **C**hemotactic **P**rotein-1 (MCP-1), through the activation of NF-κB. Taking into consideration that β-arrestins interact with IκBa, the inhibitor of NF-κB, they can hinder its nuclear translocation and gene expression[58-60]. Beta-arrestins can mediate the chemotactic effect of AngII by utilizing a p38 MAPK cascade or by using small GTPase signaling pathways, which are important in cell motility[30,61]. Arrestins interact with Arf nucleotide binding site opener and Ral guanine nucleotide dissociation stimulator (Ral-GDS), the guanine nucleotide exchange factors for the small GTPases Arf6 and Ral, respectively[62]. In this regard, β-arrestin 1 mediates the activation of RhoA by AngII, a GTPase that has been shown to play an important role in cytoskeletal structure and cell movement[31]. Thus, it seems possible that β-arrestins can induce chemotaxis in a G-independent fashion through the activation of cytoplasmic GTPases.

The emerging role of β-arrestins as scaffolding proteins that bind Raf and MEK and stimulate the phosphorylation of ERK 1/2, has received increasing attention as a signal transduction cascade in asthma, COPD and cancer. Specifically, cigarette smoke induces activation of ERK 1/2 MAPK in *in vitro* and *in vivo* experiments of COPD pathogenesis[63,64]. Nicotine is a potent inducer of ERK1/2 MAP kinase and an apoptosis suppressor of neutrophils[65]. Murine

asthma models reveal that production of the Th2 cytokines IL-2, IL-13, and IFN-γ in the airways (as represented by bronchoalveolar [BAL] fluid) is mediated via MAP kinase signaling, specifically ERK1/2 and JNK MAP kinases[66]. *In vitro* studies employing the A549 cancer cell line as well as *in vivo* studies with rats demonstrated tobacco smoke-mediated induction of the MEK-ERK 1/2 MAPK activity, which can trigger pro-proliferative or pro-inflammatory transcription factors such as c-fos, c-myc, **A**ctivator **P**rotein-1 (AP-1) and **E** twenty-six (ETS)-**Lik**e transcription factor-**1** (Elk-1) that translocate to the nucleus and enhance gene expression[63,67,68]. Air pollution and diesel exhaust particles (DEPs) has also been associated with respiratory diseases, leading to an increase in mortality[69]. DEP-evoked formation of phospho-ERK1/2 and its subsequent transfer to the nucleus depends on Ras-Raf-MEK-ERK1/2 MAP kinase signaling and upon the scaffolding action of both β-arrestins that organize these factors into a signalsome[70]. Among the genes activated is the matrix metalloproteinase-1 (MMP-1), which encodes for a zinc endopeptidase that plays a pivotal role in tissue remodeling and repair during development, in inflammation, and in the invasion, migration, and metastasis of malignantly transformed cells[71,72]. MMP-1 is involved in airway extracellular matrix degradation and pathogenetically linked to both malignant and non-malignant chronic respiratory diseases including COPD, chronic asthma, pulmonary fibrosis, emphysema, lung tuberculosis, and bronchial carcinoma[64,69,71-74]. The employing of β-arrestin-specific small interfering RNA (si RNA) led to reduced levels of phospho-ERK 1/2 and to down-regulation of MMP-1 transcription and secretion[70]. Interestingly, individuals carrying at least one copy of the –1607GG polymorphism in the 5'-promoter region of the MMP-1 gene (60-80% of the population) are more susceptible to the detrimental effects of DEP and cigarette smoke inhalation[75]. This allele is responsible for a more robust transcriptional activation of the MMP-1 gene via the Raf-MEK-ERK1/2 MAP kinase cascade, which is dependent upon both isoforms of β-arrestin. Topical delivery of β-arrestin inhibitors could arrest the MAPK signaling pathway, culminating to attenuated MMP-1 activation and providing a therapeutic benefit for patients suffering or at risk of developing respiratory diseases linked to MMP-1 dysregulation.

Beta-arrestins may also participate in the pathogenesis of pulmonary fibrosis seen in IPF or other interstitial lung diseases. In a murine model of bleomycin-induced pulmonary fibrosis, β-arrestin 1 and β-arrestin 2 knockout mice were protected from excessive collagen deposition, architectural distortion and reduced lung compliance that are known to occur after bleomycin administration[76]. BETA-arrestin-deficient lung fibroblasts revealed decreased expression of genes involved in extracellular matrix synthesis and deposition in comparison with wild-type mice, which included collagen type IV α3, collagen type V α1, laminin-α1, MMP-1 and a disintegrin- like and metallopeptidase with thrombospondin type-1 motif 2 (Adamts2). Adamts2 is a proteinase able to process pro-collagen proteins to mature collagen and is also important for collagen fibril assembly in the extracellular matrix[77]. Interestingly, neutrophil, macrophage and lymphocyte recruitment was not affected in β-arrestin deficient mice, suggesting that the protection against fibrosis development was not due to a defective inflammatory response. By functioning as multiprotein scaffolds, β-arrestins activate the Raf-MEK-ERK 1/2 MAPK signaling cascade and regulate the transcription of genes involved in extracellular matrix organization. Loss of β-arrestins limits the ability of fibroblasts to deposit excess collagen and, therefore, their pathogenic potential[76]. These findings point to a possible therapeutic effect of locally delivered β-arrestin inhibitors for IPF as well as other causes of pulmonary fibrosis.

In conclusion, β-arrestins, acting through G-protein-dependent and G-protein-independent pathways, can regulate cytoskeletal rearrangement and chemotaxis, cytokine elaboration from inflammatory cells, the progression to AHR and airway remodeling, as well as fibroblast activity and interstitial fibrosis, affecting the whole spectrum of obstructive and restrictive diseases of the lung.

The Role of β-arrestins in Oncogenesis, Tumor Cell Invasive and Metastatic Potential

Tumor cells are characterized by their aberrant growth characteristics due to loss of the physiological cell cycle regulation and by their ability to invade neighboring tissue and ultimately metastasize. The last two processes depend upon cell migration, which is a complex function controlled both by the speed and the directionality of migration that can be

triggered by external cues (i.e., chemotaxis) or because of the intrinsic property of cells to migrate (i.e., intrinsic persistence) that are in turn regulated by the Rho family of GTPases, integrins, the actin cytoskeleton, and microtubules[78,79].

Transforming growth factor-β (TGF-β) is the founding member of a superfamily of homodimeric polypeptide growth factors that have essential roles in a variety of cellular processes including development, growth control, differentiation, migration and apoptosis[80-82]. The three TGF-β isoforms that have been so far characterized, TGF-β1, TGF-β2 and TGF-β3, are encoded by distinct genes and expressed in both a tissues-specific and a developmentally regulated manner. TGF-β isoforms exert their action through binding to three high-affinity cell surface receptors, the TGF-β type I (TβRI or ALK5), type II (TβRII) and type III (TβRIII) receptors[83]. In contrast to TβRI and TβRII, TβRIII, an 849 amino acid heparan sulfate proteoglycan, functions as a co-receptor presenting TGF-β superfamily ligands to their respective signaling receptors and also has the ability to increase or decrease TGF-β signaling through mechanisms yet to be fully defined[84,85]. It is worth noting that β-arrestin 2 mediates the co-internalization of TβRII and TβRIII and downregulates TGF-β signaling[86].

TβRIII has also been established as a suppressor of cancer progression, since loss of TβRIII expression has been reported in cancers of the human kidney, prostate, ovary, pancreas and lung[87-92]. Its loss also correlates with disease progression, advanced stage or grade and a poorer outcome for patients, while increasing or restoring its expression in these cancer models decreases cancer cell motility and invasion *in vitro* and angiogenesis, invasion and metastasis *in vivo*[87-92]. Therefore, TβRIII appears to act as a suppressor of cancer cell motility.

Recent *in vitro* studies demonstrated that TβRIII and β-arrestin 2 can regulate cell migration and gene expression, both essential to tumor progression. TβRIII binds the scaffolding protein β-arrestin 2 through discrete motifs in its cytoplasmic domain and activates **C**ell **d**ivision **c**ontrol protein 42 (Cdc42)[93]. Although physiological activation of Cdc42 usually results in increased migration, constitutive Cdc42 activation by TβRIII inhibits migration. If the cells were infected with adenovirus carrying β-arrestin 2-specific siRNA, TβRIII could no longer activate Cdc42 and inhibit cell migration[93]. In terms of how β-arrestin 2 might be mediating TβRIII-stimulated Cdc42 activation to regulate the actin cytoskeleton and migration, β-arrestin 2 might be directly scaffolding TβRIII to Cdc42 to regulate its interaction with guanine nucleotide exchange factors and/or GTPase activating proteins. In addition, this interaction could be scaffolding TβRIII with other signaling molecules involved in cytoskeletal reorganization to promote actin reorganization[29]. Beta-arrestin 2 and TβRIII can also negatively regulate NF-κB signaling, again acting in a cooperative manner. NF-κB is a dimeric transcription factor that regulates genes involved in immune regulation, cell migration, inflammation and apoptosis and constitutive activation of NF-κB signaling has been reported in lung cancer, among others[94-97]. NF-κB is bound to its potent inhibitor protein IκB and sequestered in the cytoplasm. When stimulated by appropriate extracellular signals, IκB is phosphorylated by IκB kinase (IKK), which results in proteasome-mediated degradation of IκB. Once disassociated from IκB, NFκB translocates to the nucleus and activates specific target genes. Beta-arrestin 2 functions by interacting with IκB and preventing its phosphorylation by IKK, repressing NF-κB signaling[6,7]. Since TGF-β possesses tumor suppressor effects, it is possible that the TβRIII/β-arrestin 2 axis mediates its action either by binding and sequestering IκB or potentially by interfering with other yet unknown molecules of the NF-κB pathway. It is worth noting that TβRIII and βarrestin-2 can also inhibit NF-κB signaling even in the absence of TGF-β stimulation[98]. The protective effect of β-arrestin 2 against tumor progression, via its blockade of NF-κB activation, was also evident in another murine model of lung cancer[99]. Depletion of β-arrestin 2 resulted in increased expression of proangiogenic factors like VEGF, thereby promoting tumor growth and angiogenesis.

Beta-arrestins, acting again as scaffolding proteins, can also play a major role in mediating the proliferative effects of nicotine on lung cancer cells. It is well known that cigarette smoking is by far the main contributor to lung cancer[100]. It contains over 60 carcinogens, many of which are derivatives of nicotine and include molecules like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN)[101]. Nicotine exerts its cellular effects through nicotinic acetylcholine receptors (nAChRs) that are present in neuronal as well as in non-neuronal cells, like bronchial epithelial cells[102]. It was found that nicotine and structurally related car-

cinogens like NNK could induce the proliferation of a variety of small cell lung carcinoma cell lines and play a direct role in the growth progression of human lung cancers[103-105]. A study conducted in non-small cell lung cancer (NSCLC) cell lines investigated the intracellular signaling involved in the nicotine-induced cell proliferation through the activation of nAChRs[106]. The proposed mechanism involved the scaffolding action of β-arrestin 1 and the assembly of an oligomeric complex comprising the activated nAChR, β-arrestin 1 and cellular Src (c-Src) tyrosine kinase, facilitating the activation of the latter. The c-Src has well-established roles in the progression of many different human cancers[108,109] and an increase in c-Src protein levels and/or tyrosine kinase activity has been demonstrated to promote tumor cell metastasis, while inhibition of c-Src activation leads to decreased tumor cell migration and invasion. Recent evidence also showed that nicotine and NNK caused Src activation in lung cancer cells[110,111]. The next step implemented after c-Src activation, involved the activation of the Raf-1 serine/threonine kinase and its binding to the retinoblastoma (Rb) protein[107]. The Raf-1/Rb complex activated MAPK and cyclin D and cyclin E cascades that in turn recruited proliferative promoters that mediated the final step in the nicotine mitogenic signaling. Again, transfecting the cells with β-arrestin 1 siRNA completely abrogated Src activation and Rb-Raf-1 interaction and, therefore, cell proliferation after nicotine challenge[107]. It is worth noting that nicotine has been found to activate the Raf-MEK-ERK 1/2 pathway in non-neuronal tissues[112,113] and that a previous *in vitro* study had already demonstrated the role β-arrestin 1/c-Src complex plays as a mitogenic signal transducer via the Ras-Raf 1-MEK-ERK 1/2 cascade[114]. These data suggest that β-arrestin 1 can play a central role in mediating the proliferative effects of nicotine and even explaining in part the mechanism by which smokers seem to be more resistant to chemotherapy.

The β-arrestin 1/c-Src signalsome seems to mediate the cell migration effects of prostaglandins that are produced by many human cancers, and among them lung cancer[115]. These molecules are produced from membrane-expressed arachidonic acid through the enzymatic action of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The COX-2 enzyme synthesis is inducible by cytokines and growth factors and is responsible for the elevated levels of prostaglandins secreted by neoplastic cells[115]. Available clinical data are consistent with a protective effect of COX-2 inhibition, as it promotes the repression of a variety of cancer hallmark traits such as angiogenesis and metastasis[116,117]. Since the long-term use of non-steroidal anti-inflammatory drugs (NSAIDS) is associated with adverse effects[118], the molecular signals downstream of COX-2 needed to be elucidated in order to target specific steps of the COX-2 cascade. Among the prostaglandins generated by COX-2, PGE_2 is the principal one associated with cancer[119-121]. Its actions are mediated through four receptor subtypes, namely EP1-4, that belong to the GPCR superfamily. A recent *in vitro* study employing A549 lung cancer cells demonstrated the cell migration effects of PGE_2 signaled via its EP4 receptor[122]. After EP4 activation, β-arrestin 1 is recruited to the cell membrane and serves as a scaffold for c-Src leading to the formation of the β-arrestin 1/c-Src signalsome, which demonstrates increased c-Src tyrosine kinase activity. The subsequent phosphorylation of other intracellular molecules (i.e. small GTPases like RhoA) could then promote cell motility and directional migration through reorganization of the actin cytoskeleton. The use of short hairpin β-arrestin 2 RNA suppressed tumor cell migration, indicating again that β-arrestin 2 is essential for PGE_2-mediated increased cell motility[122].

Another mechanism by which β-arrestins can mediate chemotaxis and cell migration of tumor cells is through the activation of a class of receptors that belong to the GPCR superfamily, named **P**rotein-**A**ctivated **R**eceptors (PARs). Currently, there are four known PARs and they are named so because they are activated by the action of serine proteases such as thrombin (acts on PARs 1, 3 and 4) and trypsin (PAR-2)[123]. These enzymes cleave the N-terminus of the receptor, which in turn acts as a tethered ligand. In the cleaved state, part of the receptor itself acts as the agonist, causing a physiological response. In particular, PAR-2 is highly expressed in neutrophils, mast cells, and tumor cells, where it has been suggested to promote cytoskeletal reorganization[124]. The proposed mechanism for PAR-2 stimulated chemotaxis involves the activation of the receptor, which leads to an increase of the intracellular concentration of Ca^{+2}, activation of **P**rotein **K**inase **C** (PKC) and of the GTPase RhoA. PKC then phosphorylates PAR-2, promoting β-arrestin binding and subsequent internalization of the receptor into clathrin-coated pits. This endosome serves as an activating scaf-

fold for ERK 1/2 which, along with Raf-1 and other as yet undetermined factors, promotes localized activation of actin machinery, resulting in actin assembly and cell migration[125]. From a biomedical perspective, there is substantial evidence linking the PAR-2/β-arrestin 2 interaction to wound healing and tumor metastasis suggesting that PAR-2-induced, β-arrestin-dependent chemotaxis has both protective and pathophysiological roles[126,127].

Data from a recent study conducted in NSCLC patients demonstrated that β-arrestin 2 could also serve as a serum marker with prognostic implications. The serum levels of β-arrestin 2 in NSCLC patients were significantly lower than those in healthy controls while, among the cohort of NSCLC patients, β-arrestin 2 levels were higher among those in stage I than those in stage III or IV. Accordingly, among NSCLC patients, the prognosis was more favorable for those with higher serum levels of β-arrestin 2[128].

Finally, a special function of β-arrestin 1 deserves mention. Upon GPCR stimulation, β-arrestin 1 can directly translocate to the nucleus and alter gene expression by modifying the histone acetylation status. It is well known that transcription of a particular gene is dependent on the degree of histone acetylation in close proximity to this gene, especially within its promoter region. This is regulated by the opposing action of two enzymes that ultimately affect the level of acetylation of histones, namely histone acetyltransferase (HAT), which acetylates histones leading to the unwinding of chromatin and transcription of the target genes, and histone deacetylase (HDAC) that cleaves acetyl-groups and leads to chromatin packing and gene silencing[129,130]. Two possible mechanisms could be involved in β-arrestin 1-mediated histone hyperacetylation. Beta-arrestin 1 may function as a HAT activator/recruiter to increase HAT activity in the targeted chromatin regions. Alternatively, β-arrestin 1 may inhibit HDAC activity or the binding of HDAC proteins to the chromatin. The available data gathered so far point to an enhanced recruitment of HAT to chromatin mediated by β-arrestin 1, through a β-arrestin 1-dependent recruitment of a HAT co-factor named p300[131]. Therefore, β-arrestins can also act as scaffolding proteins in the nucleus, where they serve to recruit transcription factors like p300 in order to regulate gene expression. Apart from p300, **c**AMP **R**esponse **E**lement **B**inding (CREB) is also required for β-arrestin 1 mediated gene transcription. CREB is activated through a PKA-mediated phosphorylation. As a simple reminder, we refer to the activation of PKA by cAMP, the product of adenylate cyclase which is stimulated by GPCRs and their associated G proteins.

We already presented data showing that nicotine, via its nAChRs, can recruit β-arrestins and affect intracellular signaling. In addition, *in vitro* studies have demonstrated a cigarette smoke extract-mediated decrease in HDAC activity and that HDAC activity inversely correlated with COPD severity[132-134]. Its reduced activity is thought to contribute to disease pathogenesis via enhanced inflammatory cytokine production. Moreover, cigarette smoke increased intrinsic HAT activity through phosphorylation by either p38 MAPK or JNK pathways, known to be controlled by β-arrestins[132]. Thus, β-arrestin 1-dependent epigenetic regulation links a vast number of extracellular stimuli to the interior of the cell, plays a vital role in regulation of various cellular functions and reveals that such events are subjected to direct regulation by the GPCRs.

Conclusions

Beta-arrestins were long known to be regulators of GPCR function and specifically to act as negative feedback molecules of their action. Recent data demonstrate that the internalized receptor in its endosome position can still function as a mediator of intracellular signals. This is accomplished by the scaffolding action of β-arrestins and their ability to form oligomeric complexes, consisting of the internalized GPCR and other intracellular molecules.

Apart from the G-protein-dependent signaling, there are a number of actions that are mediated via G-protein-independent pathways. The ERK 1/2 MAPK, JNK3 and NF-κB signaling cascades are modulated by the scaffolding action of β-arrestins and thus physiological and pathophysiological responses like wound healing, inflammation, cell death and apoptosis, tumor invasion and metastasis can be regulated. Therefore, targeting β-arrestins with local inhibitors could serve as contributory therapy in both inflammatory lung diseases and cancer.

The receptor-β-Arrestin interactions and their consequences are presented in Table I. Figure 2 presents the intracellular pathways recruited after GPCR activation.

Table I. The receptors known to interact with β-arrestins, their combined actions and the intracellular signaling pathways modulated.

Receptor	Signaling pathway	Action
Classical GPCRs		
β2-AR, AT1R, EP1-4, PARs	G protein (Gs, Gi, Gq)	Signaling termination through: Desensitization Internalization
	ERK 1/2 p38 MAPK JNK3 c-Src family HAT/HDAC activity (nuclear trans-location of β-arrestin 1)	Scaffolding action and signalsome formation. Affects: • Cell proliferation • Chemotaxis (i.e. small GTPase phosphorylation) • Gene expression (cytokines, growth factors, chemokines, extracellular matrix proteins, peptidases etc.)
Cytokine receptors		
TβRIII (TGF-β receptor)	NF-κB Cdc42/GTPases	• Inhibition of NF-κB-dependent (IκB sequestration) • TGF-β signaling down-regulation • Regulation of actin cytoskeleton reorganization
Ion channel receptors		
nAChRs	β-arrestin 1/c-Src signalsome Ras-Raf 1-MEK-ERK 1/2 Raf-1/Rb	• Mitogenesis

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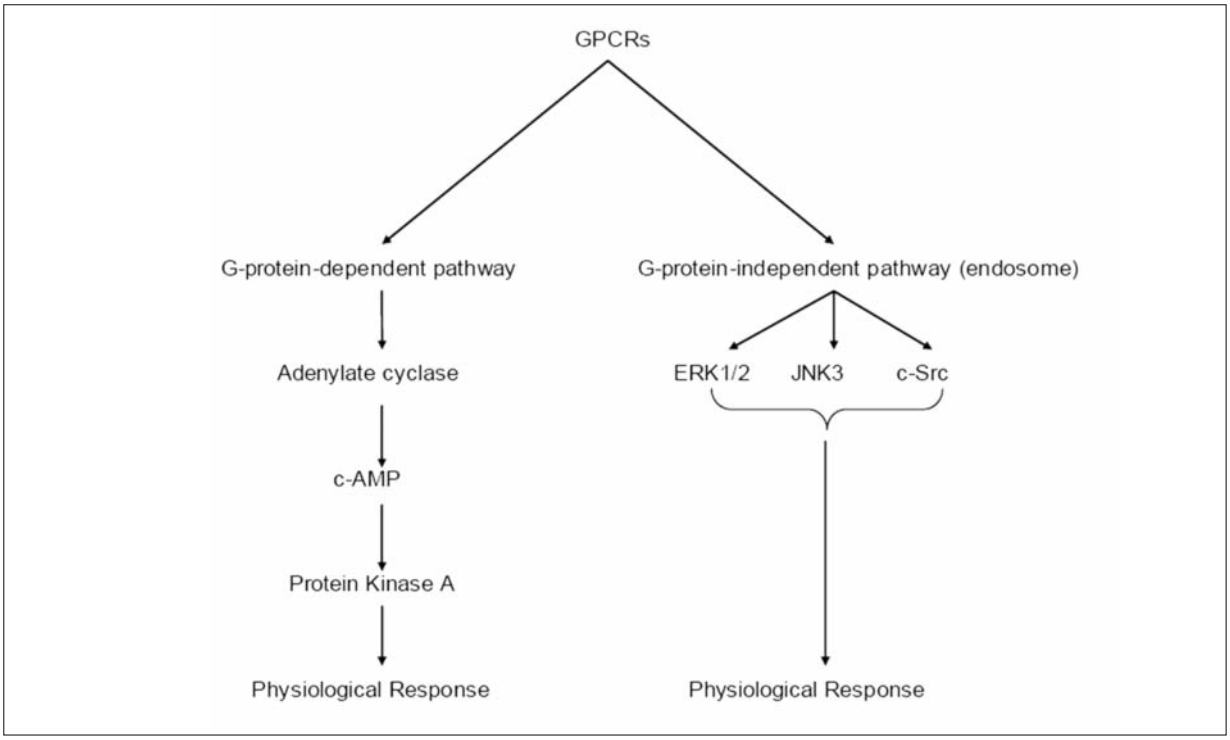

Figure 2. The intracellular signaling pathways coupled to the GPCRs.

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