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Beta-2-receptor regulation of immunomodulatory proteins in airway smooth muscle

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1. ABSTRACT

Airway smooth muscle (ASM) cells have been shown to secrete significant amounts of immunomodulatory factors (IMFs), many of which are typically ascribed to trafficking leukocytes (e.g., GM-CSF, IL-6, IL-13, and eotaxin), and may be indicative of an immunomodulatory role for ASM in control of airway function, as well as in airway diseases states associated with acute and/or chronic inflammation, such as asthma and COPD. Furthermore, epinephrine analogues such as albuterol, which ligate the G-protein coupled beta-2-receptor and have been clinically applied to promote ASM relaxation and bronchodilation in the treatment of asthma and COPD, also have been reported to downregulate IMF release by ASM, both individually and in additive fashion, in combination with corticosteroids. Based on experimental data, an inverse agonist/agonist model is proposed to explain these behaviors modeled on cell stimulatory states and G-protein coupled receptor activation. The ramifications of the model are considered in light of unexplained paradoxical clinical findings, and may provide a model for the understanding of beta-2-receptor agonist modulation of airway inflammation and function.

2. INTRODUCTION

Beta-2-receptors are ubiquitously expressed within mammalian lung and trachea, as well as within immune cells of the body (1). Of the cell types within the human lung, human airway smooth muscle (HASM) cells express a significant number of beta-2-adrenoreceptors, as many as 30-40,000 per cell (2), which are targets of naturally-occurring beta-2-receptor agonists such as epinephrine, and synthetic beta-2-receptor agonists such as albuterol. The ligation of those receptors by beta-2-receptor agonists is classically considered to be associated with HASM relaxation and subsequent airway dilation, providing an important mode of therapy in the treatment of airway obstructive diseases such as asthma and COPD, a topic covered elsewhere within this supplement series. However, it has been appreciated in recent years that airway diseases such as asthma have a significant inflammatory component, involving a host of immunomodulatory factors (IMFs) secreted by a variety of cells within the airway (3). A growing body of data and thought has emerged indicating that HASM cells are likely an important contributor to the immunodulatory milieu within the airway and lung tissue (4), and it has been

Table 1. Immunomodulatory cytokines and chemokines expressed by human airway smooth muscle cells

Cytokines	Chemokines
IL-1beta	GM-CSF
IL-5	RANTES
IL-6	MCP-1
IL-8	MCP-2
IL-11	MCP-3
LIF	eotaxin
IFNbeta	IL-8

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demonstrated that beta-2-receptor agonist and beta-2-receptor antagonist compounds produce significant, and sometimes profound effects, on the release of immunomodulatory protein factors from HASM (5-7). The release of HASM-produced IMFs is under the control of cytoplasmic and nuclear signaling pathways which are classically considered to be modulated by ligation of the beta-2-receptor, and its subsequent G-protein associated mechanisms, including signaling through cAMP (8). Thus, beta-2-receptor agonist modulation of HASM-produced factors finds relevance as a potentially important mechanism contributing to the regulation and resolution of airway inflammation associated with lung diseases such as asthma and COPD (9). Accordingly, it is the purpose of this brief review, to describe: 1) beta-2-receptor agonist-associated IMF release by HASM, 2) a theory of agonism/inverse-agonism that may partially explain behavior of beta-2-receptor agonist enantiomers in modulation of IMF release by HASM, and 3) the important interaction between enantiomeric beta-2-receptor agonists and corticosteroid compounds in the regulation of IMF release by HASM.

3. BETA-2-RECEPTOR AGONIST-MODULATED IMF RELEASE BY AIRWAY SMOOTH MUSCLE

3.1. Human airway smooth muscle as an immunomodulator

Recent evidence from Penn and colleagues (10) indicates that HASM can exhibit a profound capacity to function as an immunomodulator, such that beta-2-receptor agonist and glucocorticoid regulation of the HASM gene transcriptome may provide future targets for asthma therapy specifically targeted at HASM. Consistent with this idea, and similar to leukocytes, HASM cells release a variety of IMFs that are considered classical Th1 and Th2 cytokines and chemokines. While HASM cells do not secrete all of the interleukins within the known numerical series, they do release a substantial number of interleukins and other IMFs important in the development and resolution of airway inflammation, and possibly airway remodeling (4). The specific interleukins, and other cytokines and chemokines released by human ASM cells are shown in Table 1, and include granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-6 (4). Investigations of beta-2-receptor agonist effects on IMF release by HASM cells have focused on GM-CSF, RANTES, eotaxin, IL-6, and IL-8. (5-7, 11). Thus, although some work has shown differing mechanisms of beta-2-receptor agonist modulation of release of GM-CSF and some other IMFs (6), there will be some focus within this review on GM-CSF as a model HASM-expressed IMF regulated by beta-2-receptor-associated mechanisms, possibly having a potentially important role in the inflammation and cell proliferative processes of the airway.

3.2. Human airway smooth muscle cytokine and serum stimulatory factors

As will be illustrated within a model presented below, an important aspect of HASM cell responses to beta-2-receptor agonists involves stimulation conditions under which their studies are undertaken. A number of prior studies have focused on classical Th1-type IL-1beta or TNF-alpha stimulation of HASM (12-15), which provide a simple and straightforward model of cytokine-stimulus concentration-dependent upregulation of IMF release (12). While this solitary approach has the advantage of reducing the number of variables under consideration, the cytokine milieu with inflammation in the airway is complex, and not mono-centric. Indeed, investigations incorporating combinations of cytokine stimuli such as IL-1beta, TNF-alpha, and/or IFN-gamma have demonstrated significant enhancement of expression and release of IMFs from HASM (5, 11, 16), which likely alters the subsequent beta-2-receptor agonist behavior being studied. For this reason, some models investigating combinations of stimulatory cytokines may be more relevant to *in vivo* airway physiology, and may better enhance our translational understanding of beta-2-receptor agonist mechanisms in HASM *in vivo*.

Another aspect of experimental stimulation that affects beta-2-receptor agonist-modulated cytokine release relates to serum provision and withdrawal, which can determine the relative level of cell stimulation being studied (17, 18). Typical HASM cell culture methods utilize serum starvation for a 24-hr period to synchronize growth phase at G_0 (19). Following this interval, cells are then exposed to cytokine stimuli and the beta-2-receptor agonists being tested, with serum withheld. However, this treatment effectively minimizes cytokine production and release, such that those HASM cells are less secretory and less proliferative, and are typically considered more like constitutive cells within the airway, i.e., under nutritive cell maintenance conditions of minimal activity. On the other hand, serum repletion after the period of serum starvation induces HASM cells that are more secretory and proliferative. While providing a more complex experimental situation, serum repletion may be more representative of the stimulatory situation present with airway inflammation, and therefore, may be important in the determination of beta-2-receptor agonist-associated modulation of HASM IMF release.

3.3. GM-CSF release by human airway smooth muscle

GM-CSF is a chemokine typically associated with pro-inflammatory properties, in that it helps to sustain eosinophils (one type of granulocyte) recruited to the airways and lung parenchyma, as part of the inflammatory response within the lung (3). Given that eosinophilia is regarded as a hallmark of allergic airway inflammation associated with asthma (20), it is understandable that factors or processes which increase GM-CSF release and/or

Table 2. GM-CSF release by human airway smooth muscle cells

Cytomix + LPS	**(-)**	**(+)**
GM-CSF (pg/ml)	3.3 ± 2.1	1054.5 ± 101.5*
n	6	12

ELISA kit limit of detection = 3 pg/ml; cytomix = IL-1beta 100 U/ml, TNF-alpha 500 U/ml, IFN-gamma100 U/ml; LPS=lipopolysaccharide: 0.1 mg/ml; n = number of experiments; **P*<0.05 vs. minus cytomix + LPS. Reproduced with permission from ref. (5).

its actions would be considered pro-inflammatory, while those which reduce GM-CSF release and/or its actions, would be considered anti-inflammatory.

GM-CSF release by unstimulated HASM cells in culture is low, ranging from near or beneath detection levels (< 3 pg/ml) to approximately 50 pg/ml, as measured by conventional ELISA methods (5, 16), suggesting that GM-CSF production and secretion are low, in the absence of stimulatory cytokines associated with induction of inflammation, e.g., IL-1beta, TNF-alpha, and/or IFN-gamma, or all three as "cytomix." However, addition of these stimulatory cytokines in combination can significantly increase release of GM-CSF by HASM, (16), and the degree of this cytokine-stimulus response can be further increased 2-5-fold with the provision of serum (17, 18). Thus, as exemplified in Table 2, GM-CSF release by HASM, under these strong stimulation conditions of combined cytokines and serum, can increase significantly, from limits of detectability to a 300-fold increment, and even as much as a 1000-fold increment (5). These properties of HASM GM-CSF release in culture suggest a significant potential for upregulation of IMF release, which may indicate an important role for combinations of both stimulatory cytokines within the airway, and serum factors normally present in the human circulation, in modulating GM-CSF release by HASM *in vivo*. The classical canonical pathway through which stimulatory cytokines, such as IL-1beta and TNF-alpha, promote release of GM-CSF is that of binding to their respective membrane receptors, which subsequently activate the cytoplasmic IkappaB kinase to phosphorylate the cytoplasmic IkappaBalpha/NFkappaB complex, releasing the NF-kappaB complex, which in turn, traverses the nuclear membrane and upregulates nuclear transcription and subsequent GM-CSF protein production and release (21). As discussed below, this pathway is one target of modulation by the activity of beta-2-receptor agonists.

3.4. Modulation of human airway smooth muscle GM-CSF release by beta-2-receptor agonists

As alluded to above, the importance of GM-CSF release by HASM may be in the modulation of the airway IMF milieu with the development of inflammation, in response to a trigger stimulus. The behavior of GM-CSF release by HASM as a triggered phenomenon with provision of pro-inflammatory stimulatory cytokines supports this idea. Importantly, a number of beta-2-receptor agonists have been shown by several investigators to decrease HASM release of GM-CSF and other IMFs, under a variety of stimulus conditions, typically using racemic versions of salbutamol (albuterol), isoprenaline (isoproterenol), fenoterol, salmeterol, and formoterol (6, 7, 14, 22-24), in which there was present an equal balance (50/50 mixture) of the (R)- and (S)- enantiomers of each compound. The similarity of those findings across compounds indicated a general class effect of beta-2-receptor agonists on HASM release of GM-CSF and other IMFs. However, it is also important to note that nearly all of those racemic beta-2-receptor agonists were studied under conditions of serum withdrawal, which provides only one picture of potential mechanistic effects, and, as considered below, also has the potential for competing effects by each enantiomer within the racemate.

More recent studies of beta-2-receptor agonist enantiomers have indicated that both (R)- and (S)- isomers can have measurable effects on IMF release by HASM. Opposing effects on release of GM-CSF and IL-6 have been demonstrated for beta-2-receptor agonist enantiomers (5, 25), at concentrations in culture that would be considered reflective of those attained with inhaled albuterol treatment *in vivo*, e.g., 10 nanoM to 10 microM (26). On the other hand, those same investigations have indicated that (S)-albuterol can potentiate IMF release from HASM, clearly an opposite effect to that of the (R)-enantiomer. Those results have indicated that the previously held notion, i.e., that beta-2-receptor agonist distomers are always inert, is without foundation, and therefore must always be tested to ascertain its effects in any given experimental situation. Furthermore, (R,R)-formoterol, a beta-2-receptor agonist with two chiral carbon centers, has been shown to suppress HASM GM-CSF release even more potently and effectively than (R)-albuterol (5). As will be discussed below, those findings have further indicated that (R,R)-formoterol may be the most efficacious and potent beta-2-receptor agonist currently known, when compared with other beta-2-receptor agonists.

4. ISOMER-BASED MODEL OF BETA-2-RECEPTOR AGONIST MODULATION OF IMF RELEASE

As mentioned above, GM-CSF release from HASM cells in culture rises to significant levels with cytokine and serum stimulation, modeling conditions present with allergic airway inflammation, *in vivo*. Prior to recent work with beta-2-receptor agonist enantiomers, it was unknown as to what individual enantiomers would do to modulate HASM IMF release. As with other cytokine-secreting cells, such as T-cells and eosinophils (27, 28), in which it has been reported that structural opposite enantiomers can have opposite effects, cytokine release from HASM cells has been no exception (5, 25), again pointing to the role of HASM as an immunomodulatory cell-type that can be modulated by beta-2-receptor agonists. Importantly, it has not been well-understood how the effects of beta-2-receptor agonist enantiomers might oppose each other in modulation of functions that supposedly occur through ligation of the same membrane beta-2-receptor. In order to provide an initial framework for understanding, a model of agonism and inverse agonism may at least partially explain the behavior of beta-2-receptor agonist enantiomers, and provides a construct for

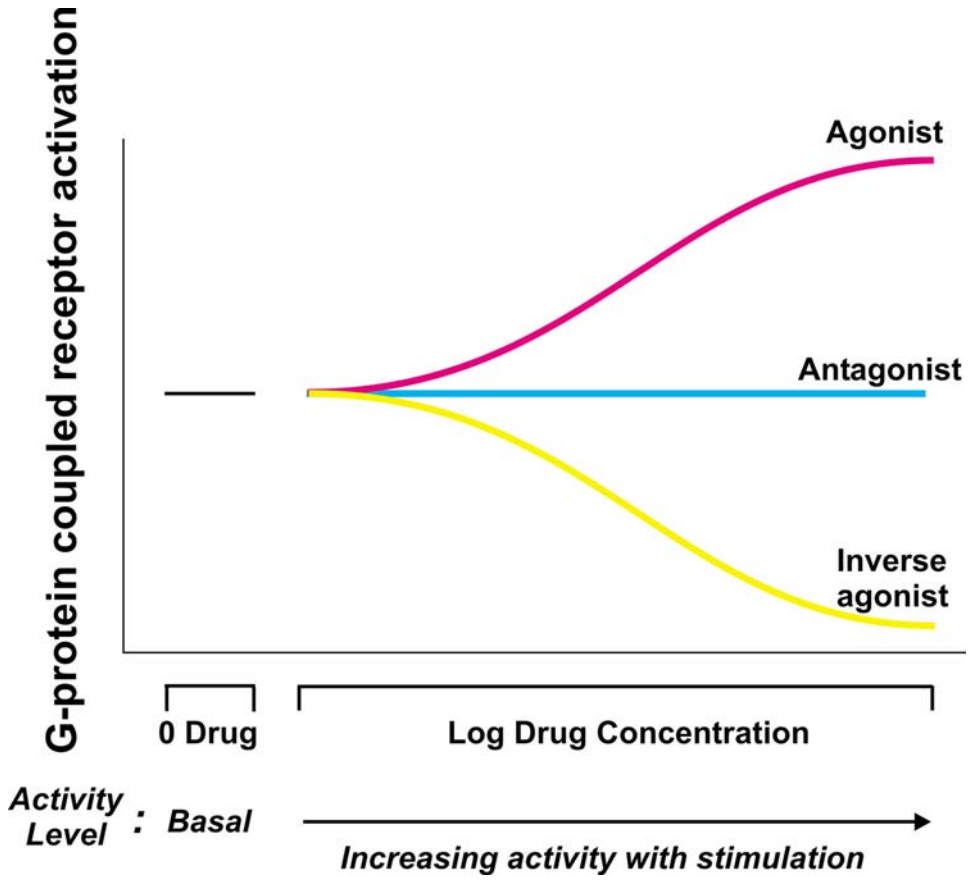

Figure 1. Model behavior of relative G-protein coupled receptor (GPCR) activation by exemplary ligands considered as agonists, antagonists, or inverse agonists, under varying conditions of cell stimulation. Ligands considered as agonists and inverse agonists demonstrate magnified opposite effects on GPCR activation as a function of increasing cell stimulation. Figure modified from Christopoulos as presented by Ellis (29) and Greener (30), with permission.

testing the effects of various beta-2-receptor agonists on HASM cytokine release under varied stimulus conditions, as well as other functions.

4.1. The GPCR activation model

Before considering the enantiomeric agonism model, it is important to understand another model, specifically, that of G-protein coupled receptor (GPCR) activation with ligand binding by Arthur Christopoulos, as outlined by Clare Ellis (29), and highlighted by Mark Greener (30). As shown in Figure 1, the GPCR activation model predicts that with increasing stimulus intensity, cells will demonstrate a measurable upregulation of GPCR activity, such that higher concentrations of drugs will be observed to have greater effects. For the case of pure agonists, this results in significantly increased GPCR activation, as shown by the upward increasing relationship within the figure. For neutral antagonists, the model predicts no measurable change in GPCR activity. However, for pure inverse agonists, the model suggests significantly decreased GPCR activation, as shown by the downward relationship. Clearly, this model predicts that under conditions of limited stimulus intensity, the difference between GPCR activity modulation by agonists and inverse agonists is limited, and may be only marginally discernable, whereas with higher stimulus intensities, the difference becomes more apparent, significantly diverging from the basal stimulus and neutral antagonist reference line. As will be discussed, this divergence of GPCR activity may be an important factor explaining the opposing effects observed of beta-2-receptor agonist enantiomers on HASM IMF release.

4.2. GM-CSF release as an output variable of an agonism/inverse agonism model

A schematic of an agonism/inverse agonism model for HASM, with GM-CSF release as the output variable that scales to relative magnitude of agonism, is shown in Figure 2, adapted from the model of Milligan and Bond (31). The scale of agonism moves to the right from zero (representing a neutral antagonist), to +1 (representing a full agonist) and also indicates relative inverse agonism, moving to the left, from zero to -1 (representing a full inverse agonist). The readout indicating the level of relative agonism or inverse agonism on this scale is the GM-CSF release measured within HASM culture supernatant, when stimulated with a strong combined stimulus of cytomix and serum. Accordingly, relative beta-2-receptor-dependent agonism is indicated by the reduction in GM-CSF release, such that more GM-CSF reduction is indicative of greater

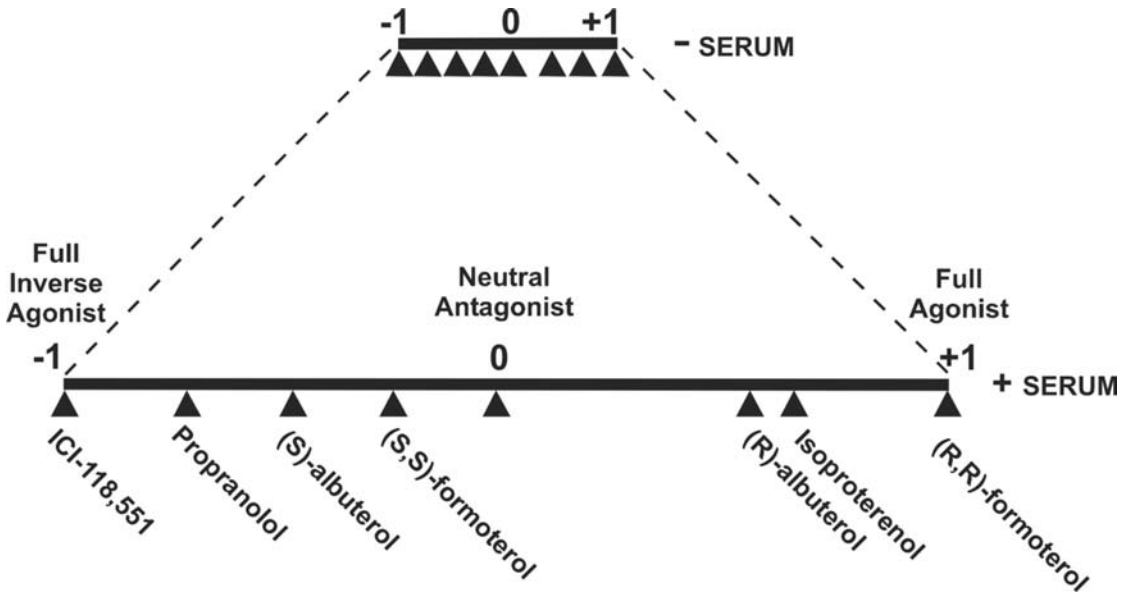

Figure 2. Agonism / Inverse Agonism model of human airway smooth muscle cell responses to beta-2-receptor ligands based on GM-CSF release data of Ameredes and Calhoun (5), with GM-CSF release as a read-out of relative ligand effects for placement on scale. In this model, relative beta-2-receptor agonism is indicated by magnitude of decreases in GM-CSF release translated into positive (agonism) values, while effects opposite to agonism are indicated by magnitude of increases in GM-CSF release translated into negative (inverse agonism) values. Expanded scale at bottom indicates separation of relative effects, with serum stimulation (+serum) of cells, while compressed scale at top illustrates decrement in separation of relative effects, in the absence of serum stimulation (-serum). Adapted after the model of Milligan and Bond from ref. (31), with permission. See text for additional details.

agonism associated with preferential binding to the beta-2-receptor by beta-2-receptor agonist eutomers. Conversely, beta-2-receptor-dependent inverse agonism is indicated by an increase in GM-CSF release, with binding of the beta-2-receptor agonist distomers to the beta-2-receptor, such that increases in GM-CSF release are associated with an opposite, or inverse effect, as compared with the agonists.

4.3. The combined model as a mechanistic postulate

By combining consideration of the agonism/inverse agonism HASM model and the GPCR activation models, a mechanism of agonist-dependent GPCR activation is suggested as the mode through which beta-2-receptor agonist eutomers may have strong and preferential effects on the modulation of HASM GM-CSF release. Accordingly, (R,R)-formoterol, is verified as the most potent agonist of those tested, demonstrating a significant ability to downregulate GM-CSF release, even more so than (R)-albuterol. For reference, the classical maximal experimental beta-2-receptor agonist, racemic isoproterenol, demonstrated a moderate amount of agonist activity in suppressing GM-CSF release from HASM, slightly more so than (R)-albuterol, as might be expected based on its classically understood effects. On the other hand, ICI-118,551, known to be a highly selective and potent inhibitor of beta-2-receptor activity (32), demonstrated an impressive ability to strongly potentiate HASM GM-CSF release, as much as 3-fold over control (5), making it an ideal candidate as a maximal reference point for inverse agonism. Again, consideration of the GPCR activation model would suggest that ICI-118,551 was highly effective at decreasing beta-2-receptor-associated GPCR activity in HASM, resulting in significant loss of the ability to downregulate GM-CSF release under conditions of intense cell stimulation. For comparison, propranolol, a classic beta-2-antagonist used both experimentally and clinically, demonstrated a moderate amount of inverse agonist-like activity on this scale, also producing significant ability to potentiate GM-CSF release in highly-stimulated HASM cells.

4.4. (S)-albuterol: the sinister half

It is within the relative agonism / inverse agonism construct, that the modus of (S)-albuterol-associated regulation of GM-CSF release by HASM may be understood. Experiments under conditions of strong stimulation (cytomix + serum) indicated that (S)-albuterol significantly upregulated GM-CSF release to a degree that approached that of propranolol (5), placing it on the inverse agonist side of the scale, opposite to that of (R)-albuterol. This was, at first, an unexpected result, due to the ongoing dogma that the distomers of beta-2-receptor agonists were considered inert. That dogma, if true, should have resulted in (S)-albuterol either being neutral, i.e., having no discernable effect on GM-CSF release and remaining at zero on the scale, or perhaps weakly agonistic, based on its 100-fold lesser avidity for the beta-2-receptor as compared to the (R)-albuterol (33). However, because the (S)-albuterol-associated increment in GM-CSF release was similar to that seen with propranolol, those results combined with other investigations have yielded some of the first clues about how distomeric beta-2-receptor

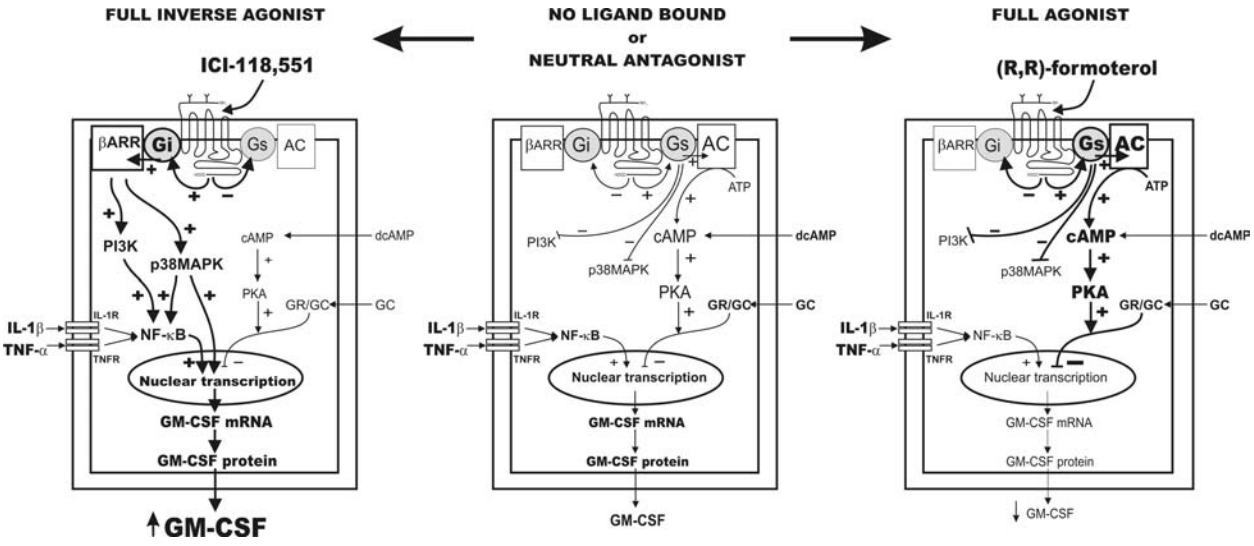

Figure 3. Schematic of potential intracellular effects of beta-2-receptor ligands modulating immunomodulatory factor release (e.g., GM-CSF) by human airway smooth muscle (HASM) cells, based on GPCR model and agonism/inverse agonism model of Figures 1 and 2, respectively. Central panel assumes a low baseline activation of beta-2-receptors in stimulated cells with either no ligand, or a pure neutral antagonist, present. Right panel illustrates effects with ligation by a full agonist such as (R,R)-formoterol, while left panel illustrates effects with ligation by a full beta-2-receptor inverse agonist, such as ICI-118,551. Relative size and bolding of text, figure components, and arrows indicate relative up- or down-regulation of GPCR-coupled processes affected by beta-2-receptor ligation by specific beta-2-receptor agonists in each case. AC= adenylyl cyclase; betaARR = beta-arrestin; Gi and Gs are inhibitory and stimulatory G-protein subunits; PKA = protein kinase A; GC = glucocorticoid, e.g., dexamethasone; GR/GC = glucocorticoid receptor-glucocorticoid complex; IL-1R = IL-1 receptor; TNFR = TNF receptor; dcAMP = dibutyryl cAMP cell-permeant cAMP inhibitor as used in experiments verifying potential role of cAMP in the modulation of GM-CSF release by human airway smooth muscle cells in culture, treated with dexamethasone (Dex) in combination with beta-2-agonist enantiomers (R)-albuterol (panel A) and (S)-albuterol (panel B). Reproduced with permission from (5).

agonists might be acting at the beta-2-receptor, or possibly elsewhere, to modulate HASM IMF release.

Work by several groups has shown that: 1) beta-2-receptor agonist (R)-enantiomers upregulate HASM intracellular stimulatory G-protein subunit (Gs), cAMP, and protein kinase A (PKA) activity (34), 2) beta-2-receptor agonist (S)-enantiomers upregulate activity of the inhibitory G-protein subunit (Gi), and downregulate cAMP and PKA activity in HASM (34), 3) addition of cell permeant dibutyryl-cAMP raises HASM intracellular cAMP, resulting in decreased release of eosinophil-activating cytokines, such as GM-CSF, RANTES, and eotaxin (5, 6), and 4) the cytokine suppressive effects of (R)-albuterol in HASM may also occur through downregulation of PI3-kinase with subsequent decreased phosphorylation of IkappaBalpha, decreasing the release of NF-kappaB from the IkappaB/NF-kappaB complex, and thus inhibiting immunomodulatory protein production (25). Therefore, based on those data, we have postulated a potential cellular scheme through which the eutomers and distomers may affect HASM cytokine release (Figure 3). In this scheme, eutomers act agonistically through ligation of the beta-2-receptor, to upregulate G_s activity, and increase PKA activity, downregulating NF-kappaB activity, with the ultimate result being downregulation of GM-CSF release, essentially considered an anti-inflammatory process. Conversely, (S)-enantiomers act in an inverse agonistic fashion, to upregulate G_i and inhibit cAMP and PKA activity, promoting a disinhibition of NF-kappaB activity, leading to increased GM-CSF production in the presence of strong pro-inflammatory stimuli like cytomix, ultimately resulting in upregulation of GM-CSF release, essentially a pro-inflammatory process.

It is important to note that these schemas are postulated to occur at moderate to high levels of cell stimulation by the combination of cytokines and serum, which are intended to represent a pro-inflammatory and pro-proliferative situation that might be present in the setting of allergic airway inflammation. Recent experiments have suggested that these conditions are necessary to observe the potent effects of beta-2-receptor agonists HASM IMF release, whereas lack of stimulation with serum does not result in profound divergence and measurable differences in GM-CSF release. Importantly, these observations are what would be predicted when the GPCR activation and agonism/inverse agonism models are considered, in the setting of mild and intense cell stimulation.

However, following up on the notion that inverse agonists can have actively opposing effects, rather than a simple inhibition of ongoing agonistic activity, there is another, as yet un-investigated possibility, that may explain how beta-2-receptor agonist distomers may promote opposing effects to beta-2-receptor agonist eutomers in HASM. In experiments in mouse embryonic fibroblasts, it

has been reported that ICI-118,551 and propranolol may exclusively rely on recruitment of beta-arrestin for their positive signaling activity (35). Furthermore, it has been pointed out that beta-arrestin can upregulate p38MAPK activity (36), which could lead to a strong promotion of GM-CSF production and release. Given that ICI-118,551 is a model maximal inverse agonist for the beta-2-receptor, and that (S)-albuterol appears to demonstrate similar characteristics to ICI-118,551 and propranolol in our data-based model, it would seem that investigation of the effects of (S)-albuterol and other beta-2-receptor agonist distomers on beta-arrestin recruitment, from an inverse agonism perspective in HASM, is warranted.

In all, the data and the models point out and support that (S)-albuterol, and possibly (S,S)-formoterol, cannot be considered inert, and can produce effects that are counter to anti-inflammatory effects of beta-2-receptor agonist eutomers. The data further suggests that the effects previously seen with racemates of beta-2-receptor agonists in HASM cells and other cells *in vitro* are due to the (R)-enantiomer, as would be expected. However, accumulating evidence on the (S)-enantiomers indicates that the presence of the (R)-enantiomers in those studies may have also negated effects of the distomers, particularly in short-term, singular cell-type culture experiments which are missing the sulphonotransferases produced by the liver and non-HASM lung cells, that preferentially metabolize the eutomers in vivo (37-40). Conversely, it is possible that the presence of (S)-enantiomers within racemates studied in vitro and in vivo may also negate beneficial effects of (R)-enantiomers, resulting in blunting of effects that would normally be attributable to (R)-enantiomers. From all of these perspectives, distomers of beta-2-receptor agonist could most appropriately be considered pro-inflammatory rather than inert, and potentially counter-productive to the clinical therapeutic intent of beta-2-receptor agonist administration in asthma and COPD.

The idea that receptor-ligating isomers can have differing effects within the same receptor population is not new, and has practical and experiential evidence within human investigation and history, in its support. For example, the difference in taste between spearmint leaves which contain L-carvone, and caraway seeds, which contain D-carvone and taste bitter, is due to isomers of the same chemical structure having very different results when coming into contact with taste receptors on the tongue. D-limonene smells piney like turpentine, while L-limonene smells like oranges, again, due to isomer structural differences resulting in an odor sensation difference, when in contact with receptors in the nose. Another example is thalidomide, with one isomer inducing the relinquishment of nausea, but the other isomer being teratogenic, and causing fetal defects *in utero*, by reducing growth of blood vessels. Similarly, naproxen is an isomer used in treatment for arthritis, while its other isomer causes liver dysfunction. Thus, inverse agonism with isomers of beta-2-receptor ligands has a similar conceptual basis, and it is the application of this concept to beta-2-receptor functional output that bears further consideration, as novel therapies for asthma and COPD continue to be developed.

5. BETA-2-RECEPTOR AGONIST /STEROID COMBINATIONS AS IMF CO-MODULATORS

5.1. Treatment-based combinatorial rationale

It has long been known that corticosteroids have potent anti-inflammatory properties *in vivo* and *in vitro* (41-44). In recent years, drug combinations incorporating beta-2-receptor agonists and corticosteroids have become popular in the treatment of asthma and COPD, due to their efficacy in targeting what where originally thought to be separate pathways, in order to gain a therapeutic advantage. For example, in treatment of diseases such as asthma, the notion was that treatment of the airway inflammatory component with an anti-inflammatory steroid, with simultaneous treatment of airway constriction using a beta-2-receptor agonist to promote relaxation of HASM. Admittedly, this notion primarily focuses on the contractile properties of HASM, but it should now be clear that increased attention should be given to the immunomodulatory function of HASM within this scenario. In support of this idea, but beyond expectations of simple addition, it has been consistently reported that the combination is therapeutically more effective than either separately (45-47). The success of combination therapy in treating asthma, such as that with the formulation of salmeterol + fluticasone (48), highlights the importance of understanding these mechanisms, and is varyingly considered a product of facilitative, additive, and/or synergistic of a facilitative, additive, and/or synergistic cellular mechanisms between these two important classes of compounds within immunomodulatory cells of the lung. Thus, the interaction of beta-2-receptor agonists and steroids has become an important area of research with regard to both mechanisms of action, and asthma/COPD treatment, in recent years (49).

5.2. Evidence for associative immunomodulatory mechanisms in human airway smooth muscle

Studies in immunomodulatory T-cells have indicated that beta-2-receptor agonist and corticosteroid combinations synergistically reduce production of Th-1 cytokines (50), effects which can occur through increased expression of IkappaBalpha, which in turn, limits NF-kappaB activation, all of which are associated with enhanced nuclear translocation of glucocorticoid receptor (50, 51). A similar synergistic behavior through increased expression of IkappaBalpha has been demonstrated in HASM, treated with beta-2-receptor agonists such as salbutamol and salmeterol, in combination with either dexamethasone or fluticasone, which inhibited IL-8 release (24). Other studies in HASM indicate that beta-2-receptor agonists inhibit both TNF-alpha-associated histone H4 acetylation and NF-kappaB p65 subunit binding, resulting in decreased eotaxin release, similar to corticosteroids (22). Importantly, steroids may facilitate the actions of beta-2-receptor agonists through maintenance of beta-2-receptor numbers and avoidance of desensitization, by increased transcription of the beta-2-receptor gene, increased expression of beta-2-receptors on the membrane, and decreased uncoupling of beta-2-receptors from Gs through inhibition of G-regulated kinase (GRK)-2 expression (49, 52-54). However, it bears noting for future studies that recent work also suggests that beta-2-receptor agonists may

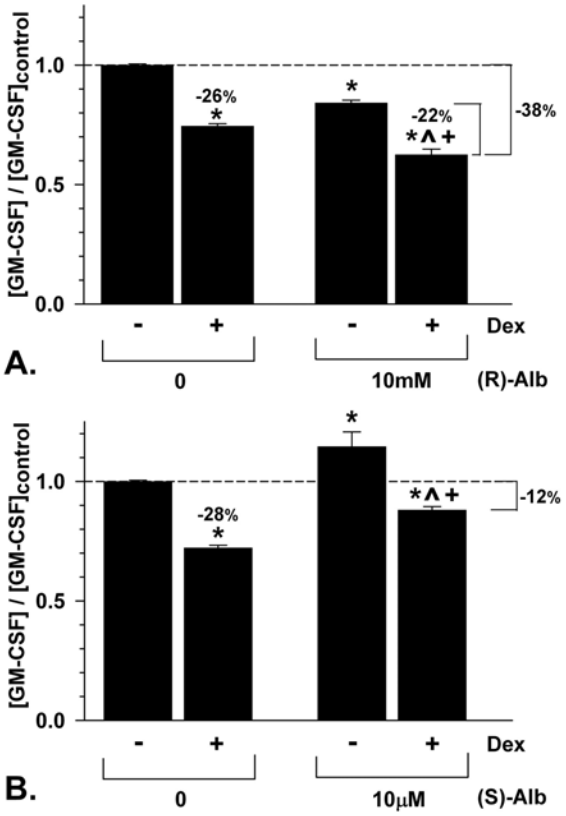

Figure 4. GM-CSF release by human airway smooth muscle cells in culture, treated with dexamethasone (Dex) and beta-2-receptor agonist enantiomer combinations. GM-CSF expressed as ratios relative to –Dex, 0 enantiomer control (range: 1800-2700 pg/ml); percentage change values as shown. **P*<0.05 vs. –Dex, 0 agonist (no drugs) control; $^{+}$*P*<0.05 vs. –Dex, 10 microM agonist; ^*P*<0.05 vs. +Dex, 0 agonist; n = 4-10 experiments/bar. Reproduced with permission from Ameredes and Calhoun, ref. (5).

exert their effects on IMF release through other "non-generic" effects on NF-kappaB (55), as well as non-G-protein-associated pathways (56). Thus, we are just beginning to understand the interleaved and complimentary nature of the beta-2-receptor agonist and steroid pathways within HASM, and their potential as future targets for therapeutic manipulation.

5.3. Isomer enhancement and suppression of steroid effects

It stands to reason that, given the characteristics of beta-2-receptor agonist eutomers and distomers outlined above, it is prudent to consider how the anti-inflammatory effects of steroids in HASM might be affected by co-administration with (R)- and (S)-enantiomers of albuterol and formoterol. As illustrated in Figure 4, one study indicated that co-administration of dexamethasone with (R)-albuterol results in an additional reduction of GM-CSF release in HASM, suggesting an enhancement of the anti-inflammatory effect of the steroid by a eutomer (5), and reminiscent of the effects reported in T-cells (50). Conversely, co-administration of dexamethasone with (S)-albuterol had an opposite effect, such that the steroid-dependent reduction in GM-CSF release was negated and reversed, suggesting an abrogation of the anti-inflammatory effect of the steroid by a distomer (5). These effects would be consistent with the consideration of (S)-albuterol as an inverse agonist, as put forth within the models above, again providing some framework for our understanding of the effects of distomers on HASM, and potentially their interaction with anti-inflammatory steroids.

6. SUMMARY AND PERSPECTIVE: SINISTER EFFECTS ON AIRWAY FUNCTION?

In summary, beta-2-receptor agonists can modulate IMF release from HASM, dependent on cell stimulation conditions, which has relevance in the setting of airway inflammation. Furthermore, the results of basic cellular investigations indicate that isomers of beta-2-receptor agonists have effects which oppose each other. As would be expected, eutomers, similar in structure to natural or endogenous epinephrine, have beneficial, anti-inflammatory effects. However, distomers, with the opposite molecular configuration are not just inert; in contrast, they can have effects which oppose their eutomer counterparts. A model of IMF release, as a function of beta-2-receptor cell signaling, suggests that distomers can act to produce effects consistent with the concept of inverse agonism, providing a novel construct from which to understand and investigate beta-2-receptor function. Such a construct may also provide insight into mechanisms as to how combinations of various beta-2-receptor agonists and steroids affect airway function.

With the perspective regarding distomers cited above, questions arise about the mechanistic source of certain findings of prominent clinical studies involving racemic beta-2-receptor agonists. For example, the demonstration of a significantly greater loss of lung function upon withdrawal of regular racemic albuterol in asthmatic Arg-16 homozygotes, which remains unexplained, but was only mitigated with ipratropium (57-59), suggested the possibility of an unmasked effect of the (S)-albuterol portion of the racemate. Consistent with those findings, at least one clinical study, modeling inhaled corticosteroid withdrawal, reported that time to loss of control, which included measures of airway inflammation, was approximately 50% more rapid in asthmatics maintained on racemic albuterol (9 days), as compared to racemic albuterol + ipratropium (17 days) (60). Finally, recent concern has surrounded the use of long-acting beta-2-receptor agonists in the treatment of asthma, in part due to studies of racemic salmeterol in which an unexpected number of deaths were reported (61). Furthermore, it has been shown that steroid withdrawal with racemic salmeterol maintenance can lead to a significant deterioration in asthma control (62). Those findings and others have prompted a recommendation for limitation of the long-term therapeutic use of salmeterol, and in fact, all long-acting beta-2-receptor agonists including formoterol, such that they be used *only* in conjunction with anti-inflammatory corticosteroids (63), in order to mitigate long-acting beta-2-receptor agonist-associated problems

that might arise with increased airway inflammation (64-66). However, it is worth noting that each of the investigations on which this recommendation is based utilized racemic beta-2-receptor agonists, and can be considered to have a significant portion of their origin within effects on HASM, perhaps through an association of immunomodulatory and contractile properties of that cell type. In all, the findings and models presented above suggest that, while beta-2-receptor agonist and steroid immunomodulatory pathways in HASM are strongly linked, further consideration of the therapeutic use of racemic mixtures versus enantiomerically-pure versions of both short-acting and long-acting beta-2-receptor agonists is necessary, as treatment targets for asthma and COPD continue to be refined and developed.

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Abbreviations: AC: adenylyl cyclase; Arg: arginine; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; COPD: chronic obstructive pulmonary disease; dcAMP: dibutyryl cAMP; Dex: dexamethasone; ELISA: enzyme-linked immunosorbent assay; GM-CSF: granulocyte-macrophage colony stimulation factor; GPCR: G-protein coupled receptor; GC: glucocorticoid; GR: glucocorticoid receptor; GR/GC: glucocorticoid receptor-glucocorticoid complex; GRK: G-regulated kinase; HASM: human airway smooth muscle; IL-1R: IL-1 receptor; IMF: immunomodulatory factor; LIF: Leukemia inhibitory factor; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; MCP: methyl-accepting chemotaxis protein; NFkappaB: nuclear factor kappa B; PI3K: phosphatidylinositol 3-kinase; PKA: protein kinase A; RANTES: regulated on activation normal T cell expressed and secreted; (R): rectus; (S): sinister; Th-1: T-helper cell 1; Th-2: T-helper cell 2; TNFR: TNF receptor;

Key Words: Albuterol, Antagonist, Beta-Arrestin, Beta-Receptor, Formoterol, GM-CSF, Levalbuterol, Ligand, Inverse Agonism, Racemic Mixture, (R)-Albuterol, (S)-Albuterol, Review

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