Molecular interactions between glucocorticoids and long-acting β_2-agonists

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β_2-Adrenergic receptor agonists and glucocorticoids are the two most effective treatments for asthma, and used in combination they are more effective than either alone. Glucocorticoids mediate their anti-inflammatory effects through the action of activated glucocorticoid receptors (GRs), with the level of activity being related to the number of nuclear receptors. Glucocorticoids can upregulate the synthesis of several genes in human lung cells through interaction with specific DNA binding regions (glucocorticoid response elements) within the promoter region of glucocorticoid-responsive genes. Many of the down-regulating effects of GRs on the synthesis of cytokines and other inflammatory mediators are due to repression of other transcription factors, such as activator protein-1 and nuclear factor κB. GR functions such as nuclear localization and gene activation can be regulated by phosphorylation status. Long-acting β_2-agonists may affect GR nuclear localization through modulation of GR phosphorylation and furthermore through priming of GR functions within the nucleus by modifying GR or GR-associated protein phosphorylation. Glucocorticoids in turn may regulate β_2-adrenergic receptor function by increasing its expression, acting through glucocorticoid response elements, and, importantly, by restoring G-protein–β_2-receptor coupling and inhibiting β_2-receptor down-regulation, thereby preventing desensitization. (J Allergy Clin Immunol 2002;110:S261-8.)

Key words: *Phosphorylation, mitogen-activated protein kinase, nuclear translocation, gene induction*

Long-acting β_2-adrenergic receptor agonists (LABAs) and glucocorticoids are the two most effective treatments for asthma and are more potent in combination than either drug alone.[1,2] Whereas glucocorticoids are used to treat airway inflammation, LABAs are used as bronchodilatory agents to bring rapid relief of airway bronchoconstriction. It is unclear whether LABAs have major anti-inflammatory actions in themselves in vivo as opposed to their potent effects in vitro,[3] suggesting that the added benefit of combination therapy probably relates to cross talk between the two drugs. This review summarizes the interactions between these drugs at the biochemical and molecular levels and discusses the possible effects of LABAs on glucocorticoid function and vice versa.

Abbreviations used
AP-1: Activator protein-1
β_2AR: β_2-Adrenergic receptor
cAMP: Cyclic AMP
CBP: CREB binding protein
CREB: Cyclic AMP response element binding protein
ERK: Extracellular signal-regulated kinase
GR: Glucocorticoid receptor
GRE: Glucocorticoid response element
LABA: Long-acting β_2-adrenergic receptor agonist
MAPK: Mitogen-activated protein kinase
NF-κB: Nuclear factor κB
PKA: Protein kinase A
PKC: Protein kinase C

MECHANISMS OF GLUCOCORTICOID ACTION

Endogenous glucocorticoids regulate the body's normal reactions to stress, preventing those reactions from overshooting and threatening homeostasis.[4] Thus, many of the physiologic and pharmacologic effects of glucocorticoids may be secondary to modulation of the action of numerous intercellular and intracellular mediators, including other hormones, prostaglandins, lymphokines, and bioactive peptides.[5] Glucocorticoids act by influencing transcription of target genes.[6] Glucocorticoids freely diffuse into the cell and bind to the glucocorticoid receptor (GR), which is held in an inactive form within the cytoplasm by a number of molecular chaperones, including heat shock protein 90.[7] On ligand binding, the GR undergoes a conformational change, resulting in dissociation of heat shock protein 90, unmasking of a nuclear localization signal, and nuclear translocation.[8] Within the nucleus, GR may either bind to specific glucocorticoid response elements (GREs) in the promoter region of steroid-sensitive genes or interact with, and inhibit, proinflammatory transcription factors, such as activator protein-1 (AP-1) and nuclear factor κB (NF-κB).[8] GR-GRE interaction leads to recruitment of cofactors, including

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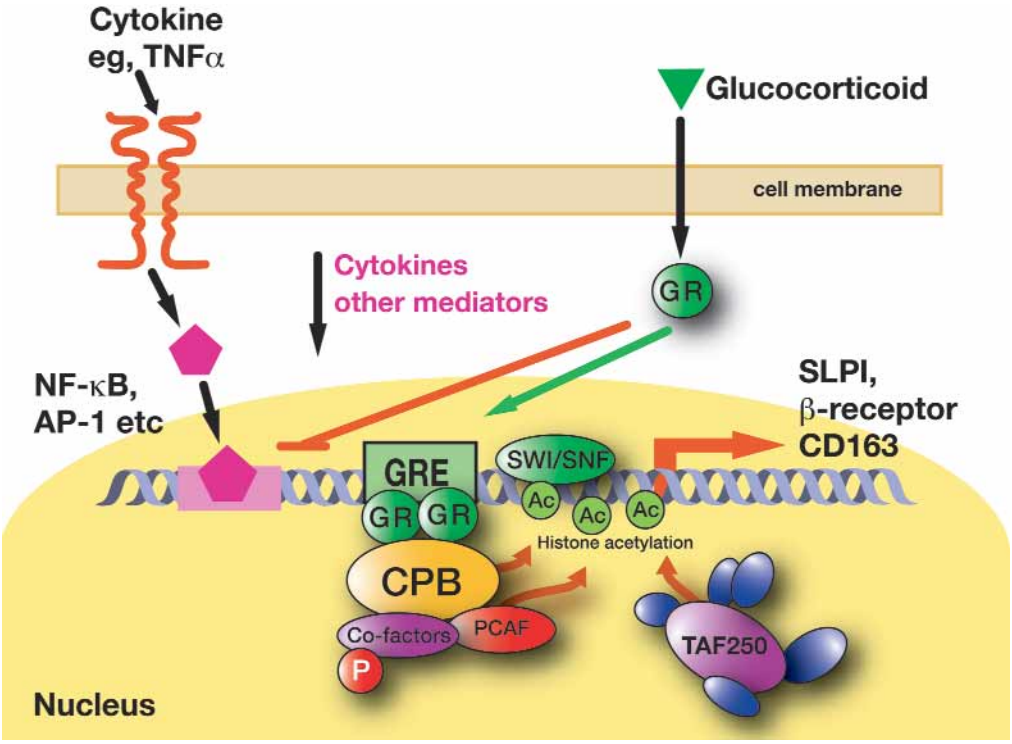

FIG 1. The mechanism of glucocorticoid action. Glucocorticoids freely diffuse from the circulation across cell membranes, where they interact with a GR. On ligand binding the receptor is activated and translocates to the nucleus, where it can bind as a dimer to specific DNA sequences *(GREs)* upstream of the start site of transcription. This induces recruitment of cofactors such as CBP and p300/CBP-associated factor *(PCAF),* which induce histone acetylation *(Ac)* and further recruitment of chromatin remodeling factors, such as the switch/sucrose nonfermentable complex *(SWI/SNF),* and finally induce recruitment of the basal transcription complex, including TAF250, and modulation of target gene transcription. The activity of many of these proteins is regulated by phosphorylation *(P).* Alternatively, glucocorticoids may act by inhibiting the abilities of other transcription factors activated by cytokines, such as AP-1 and NF-κB, to induce proinflammatory gene transcription. In this instance GR acts as a monomer. *SLPI,* Secretory leukocyte protease inhibitor.

CREB binding protein (CBP), modulation of chromatin structure, and subsequent induction of gene transcription.[8] Repression of AP-1 and NF-κB involves recruitment of histone deacetylases and other repressor proteins, resulting in inhibition of AP-1–directed and NF-κB–directed gene expression.[9,10] CBP interacts with a variety of different transcription factors and with components of the basal transcriptional machinery.[11] Thus multiple coactivators may form a ternary complex on DNA to modulate transactivation mediated by a single DNA-binding transcription factor (Fig 1).[10]

In the resting state the predominant subcellular localization of GR is within the cytoplasm. This is a result of an active process, in that inhibition of nuclear export results in a nuclear unliganded GR.[12] This suggests that the unliganded GR is able to shuttle between the cytoplasm and the nucleus.

EFFECTS OF GR PHOSPHORYLATION STATUS ON GR FUNCTION

GR is a phosphoprotein containing 64 potential phosphorylation sites,[13] including those for extracellular signal-regulated kinase (ERK, 8 sites), p38 mitogen-activated protein kinase (MAPK, 1 site), glycogen synthase kinase-3 (8 sites), protein kinase C (PKC, 9 sites), and protein kinase A (PKA, 8 sites). Importantly, GR has 3 ERK binding sites (Fig 2). After DNA binding, GR interacts with a number of cofactors and the basal transcription complex to regulate GR-responsive genes, many of which are also phosphorylated. The role of receptor phosphorylation in receptor function is controversial, however, because promoter complexity and context may affect the ability of phospho-GR to regulate transcription. Evidence obtained during the past 10 years clearly suggests that altered GR phosphorylation status can affect GR-ligand binding,[14] heat shock protein 90 interactions,[15] subcellular localization, nuclear-cytoplasmic shuttling,[16,17] and transactivation potential.[18]

Inhibition of serine-threonine protein phosphatase type 5 with antisense oligonucleotides has been shown to induce unliganded GR-GRE binding and transactivation activity and to enhance ligand-induced DNA binding and transactivation 10-fold.[19] Okadaic acid, a protein phosphatase type 1 and protein phosphatase type 2A inhibitor, has the same effects, apparently because of an accumulation of nuclear GR, and its actions may require phosphorylation of GR-associated proteins.[19]

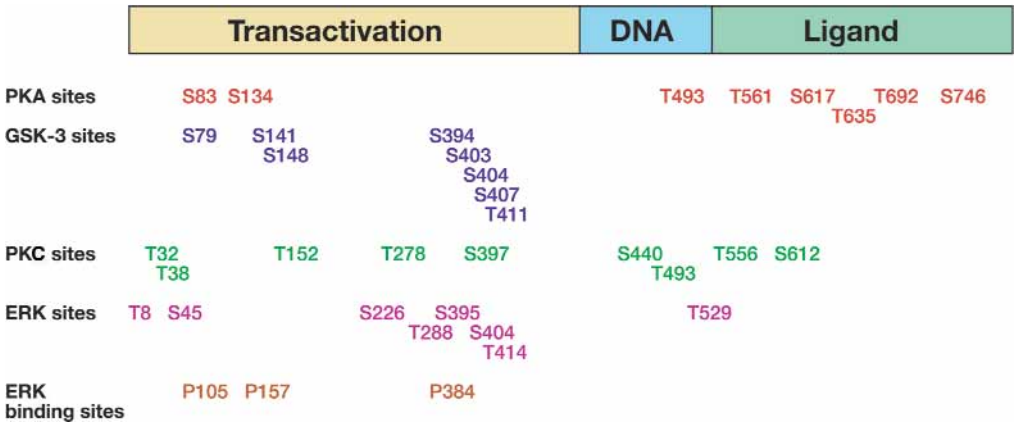

FIG 2. Potential phosphorylation sites on GR. Some of the possible PKA, glycogen synthase kinase-3 *(GSK-3),* PKC, and ERK phosphorylation sites are indicated. Also shown are the 3 potential ERK docking sites that are essential if a protein is to act as an ERK substrate.

Inhibition of tyrosine phosphorylation by genestein and tryphostin AG126 can also enhance GR nuclear export, although this may not be due to direct actions on GR.[20] Further evidence that GR phosphorylation is important is that ligand binding induces GR hyperphosphorylation at 7 sites, which in turn regulates transactivation and reduces nonspecific DNA binding.[21] Thus global changes in GR charge may affect its function as well as specific phosphorylation events (Fig 3).

Many studies have shown that a cell's response to glucocorticoids depends on the stage of the cell cycle, with cells being less sensitive during G2/M.[22] In a series of extensive experiments over many years, Munck and DeFranco[23,24] have shown in rat GR that this altered glucocorticoid sensitivity is due to altered GR phosphorylation status during cell cycle progression and targeting of specific serine residues 224 and 232. Notably, the loss of GR function in G2/M is associated with global increases in basal GR phosphorylation status and a failure to induce further GR phosphorylation.

Recent evidence from Rogatsky et al,[25,26] who examined the rat GR, suggests that Jun-N-terminal kinase and glycogen synthase kinase-3 are both able to phosphorylate GR directly and decrease GRE transactivation. Phosphorylation occurs at distinct sites (S224, S246, and T171) that may be targets for other kinases and phosphatases, with the final functional outcome dependent on the overall pattern of phosphorylation. For example, phosphorylation of rat GR on T171 by glycogen synthase kinase-3 reduces GRE transactivation without affecting repression of an AP-1–driven promoter.[25]

It has also become evident that ERK is involved in several of the mechanisms of GR action. Vanadate, a transition metal oxyanion similar to molybdate, has been shown to induce time-dependent and concentration-dependent increases in both ERK and nonreceptor tyrosine kinase activity that result in an increase in unliganded receptor GRE binding and markedly enhance transactivation in the presence of the ligand.[27] The cyclooxygenase-2 inhibitor nimesulide is also able to enhance ERK activity, GR phosphorylation, GR-GRE interactions, and transcriptional activity without affecting GR expression or nucleocytoplasmic shuttling. Many of the actions of nimesulide are thought to be mediated through these mechanisms.[28]

Recent data from Wallace and Cidlowski[29] have delineated another role for GR phosphorylation. Decreased phosphorylation in the mouse GR decreases transactivation of a 2 × GRE promoter and alters the localization of the unliganded receptor without affecting that of the liganded GR. Interestingly, GR half-life is greatly increased with decreased phosphorylation, suggesting that phosphorylation is involved in receptor turnover and that phosphorylation could target the receptor for hormone-mediated degradation. As such, phosphorylation-induced targeting of GR for ubiquitination and proteosomal degradation may play an important role in overall GR responsiveness.

MECHANISMS OF β-AGONIST ACTION

Ligand binding to the β_2-adrenergic receptor (β_2AR) results in activation of receptor-associated G_s proteins and enhanced coupling with adenylyl cyclase.[3] The coupling of activated G_s and adenylyl cyclase leads to enhanced production of cyclic AMP (cAMP) and subsequent activation of cAMP-dependent PKA, which then phosphorylates and thus inactivates myosin light chain kinase, preventing myosin phosphorylation. Concomitant activation of calcium-magnesium exchange ATPases in the endoplasmic reticulum and plasma membrane[3] decreases ionic calcium levels, thereby reducing calcium-dependent actin-myosin interactions and leading to relaxation of airway smooth muscle.

β_2-Agonists may also influence gene transcription through elevation of cAMP and activation of PKA.[30] cAMP mediates the hormonal stimulation of a variety of eukaryotic genes through a conserved cAMP response element.[30] Transcriptional induction by cAMP is rapid, peaking at 30 minutes and declining gradually over 24

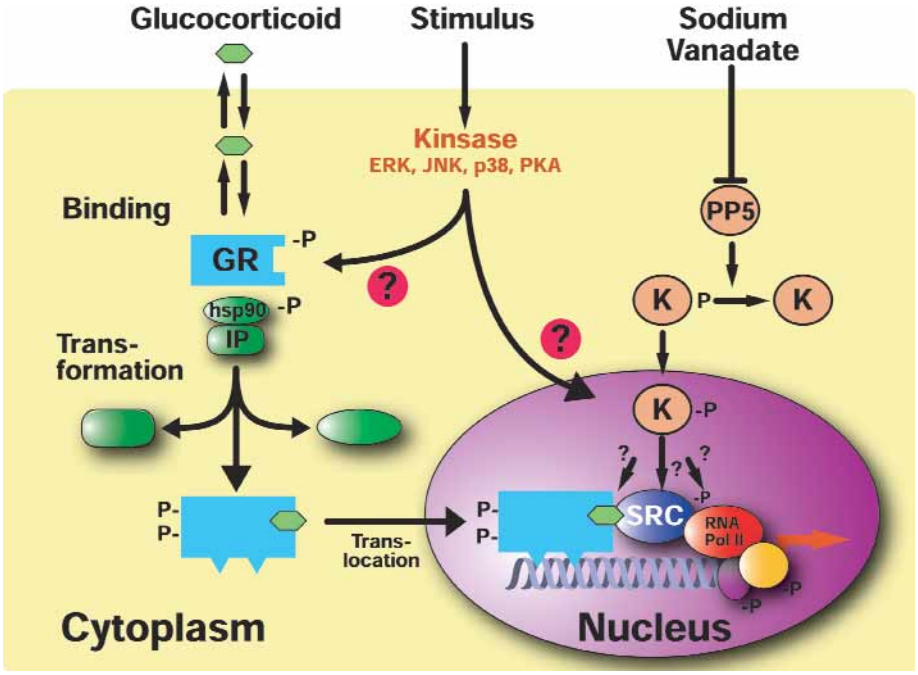

FIG 3. Inhibition of protein phosphatase type 5 *(PP5)* by vanadate modulates GR phosphorylation and activity. Glucocorticoids freely diffuse across the cell membrane to interact with the cytosolic GR. This is held within the cytoplasm as an inactive complex by chaperones such as heat shock protein 90 *(hsp90)* and immunophilin *(IP)*. Binding of the ligand induces GR hyperphosphorylation, dissociation from heat shock protein 90 and immunophilin, and nuclear translocation. The phosphorylation status of the chaperones may also alter during these steps. Within the nucleus, phosphorylated GR binds to DNA at a GRE, leading to recruitment of cofactors such as steroid receptor coactivator *(SRC)* and activation of RNA polymerase II *(RNA Pol II)*. The activities of these factors are altered by phosphorylation. Modification of GR phosphorylation may be mediated through extracellular stimuli that activate intracellular kinases, such as PKA, PKC, ERK, and Jun-N-terminal kinase. These kinases may either phosphorylate GR directly or act indirectly through GR-associated factors. In addition, the activity of phosphatases can regulate GR function. Inhibition of protein phosphatase type 5 by vanadate leads to activation of a GR-kinase or GR-associated-factor–kinase, resulting in enhanced GR transcriptional activity. (Calzi SL, Periyasamy S, Li DP, Sánchez ER. Vanadate increases glucocorticoid receptor-mediated gene expression: a novel mechanism for potentiation of a steroid receptor. J Steroid Biochem Mol Biol 2002;80:35-47. With permission.)

hours.[30] This burst in transcription is resistant to inhibitors of protein synthesis, suggesting that cAMP may stimulate gene expression by inducing the covalent modification rather than by inducing de novo synthesis of specific nuclear factors. Treatment of cells with cAMP causes translocation of the catalytic subunit of PKA to the nucleus,[30] where it phosphorylates serine 133 on CREB, enhancing its DNA-binding and transactivating activity.[30] CREB mediates its transactivating abilities through the associated CBP that transduces the CREB signal to the transcription initiation complex. Activated CREB may persist for prolonged periods within the nucleus, and, therefore, even a brief exposure to β_2-agonist may lead to a prolonged effect on transcription.

cAMP may also interfere with the effects of PKC activation through inhibition of MAPK.[31] More recently, however, it has been reported that activation of the β_2AR can also lead to coupling to G_i, resulting in stimulation of the ERK MAPK pathway.[32] PKA-mediated β_2AR phosphorylation uncouples G_s from the β_2AR and enables $\beta\gamma$-subunit–mediated G_i coupling, leading to Src, SoS, and Ras stimulation and ultimately to ERK MAPK activation (Fig 4). Thus the balance between these two mechanisms of G-protein subunit coupling regulates the MAPK response to β-agonists.[33] Collins et al[34] recently reported that activation of the β_2AR can also induce p38 MAPK activation in a manner similar to that seen with ERK MAPK activation.

The rate of transcription of the β_2AR gene is increased in response to β-agonist stimulation of the receptor at the cell surface. This positive autoregulation of the β_2AR gene appears to occur through receptor-mediated stimulation of adenylyl cyclase, with consequent activation of CREB and stimulation of β_2AR gene transcription.[35] However, most long-term exposure to β-agonists results in decreased mRNA in cell lines and in lung in vivo.[36] This reduced expression of β_2AR is due to reduced gene transcription and is associated with a reduction in CREB activity, and it may be related to receptor desensitization or internalization.[37] The initial step in β_2AR desensitization is phosphorylation by PKA, PKC, or the G-protein–coupled receptor kinase. This causes β-arrestin binding to the β_2AR, resulting in steric inhibition of G_s-coupling and reduced

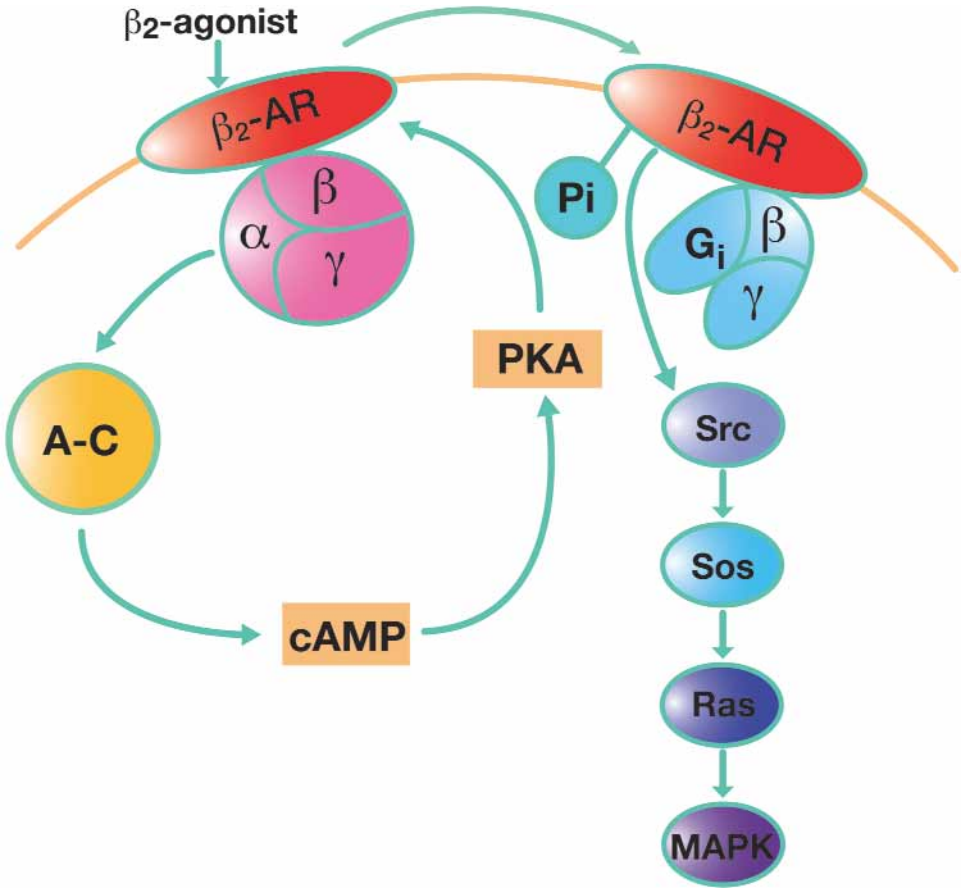

FIG 4. β_2-Agonists can induce both PKA and MAPK activation. Activation of the β_2ARs by β-agonists causes receptor-associated G_s proteins (α, β, and γ subunits) to be activated and couple with adenylyl cyclase *(A-C).* The coupling of activated G_s and adenylyl cyclase leads to enhanced production of cAMP and subsequent activation of cAMP-dependent PKA. Activation of PKA may lead to β_2AR phosphorylation and uncoupling of G_s from the β_2AR. This enables βγ-subunit–mediated G_i coupling, leading to stimulation of the ERK MAPK pathway. ERK MAPK activation occurs subsequent to Src, SoS, and Ras stimulation. (From Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. Nature 1997;390:88-91. With permission.)

cAMP production. The receptor may be subsequently internalized through clathrin-coated pits and degraded or dephosphorylated and recycled.[3] Pro-inflammatory cytokines such as IL-1β and prostaglandin E_2 may also promote β_2AR desensitization.[3]

EFFECTS OF LABA ON GR FUNCTION: POSSIBLE ROLE OF GR PHOSPHORYLATION

In an important in vitro study, Eickelberg et al[38] found that in primary human lung fibroblasts and vascular smooth muscle cells both salbutamol and salmeterol could induce GR nuclear translocation and enhance GR-GRE binding in the absence of ligand. Translocation of GR by β_2-agonists was less effective than that seen with dexamethasone and was PKA dependent. This study has since been confirmed in preliminary reports in vivo,[39-41] which have indicated that salmeterol can induce GR nuclear translocation and that this may prime the receptor to be more responsive to a concomitant or subsequent challenge with glucocorticoid. Evans and Bloom have found that in BEAS-2B cells salmeterol can enhance fluticasone activation of a GRE-luciferase reporter gene 50% above the maximal level achieved with fluticasone (10^{-6} mol/L) alone (Bloom J, University of Arizona College of Medicine, Tucson, AZ, personal communication). Salmeterol also enhanced fluticasone repression of NF-κB reporter gene. This group has also found that salmeterol caused a rapid increase in GR phosphorylation at serine residues 141, 211, and 226 (Bloom J, University of Arizona College of Medicine, Tucson, AZ, personal communication). Phosphorylation of GR and GR-associated factors may alter GR nuclear translocation of the unliganded receptor, but more importantly it may alter cofactor recruitment. In addition, the activity of these cofactors may be enhanced by PKA and MAPK pathways.[42] Functionally, this enhances GR responsiveness to both transactivation and transrepression actions and leads to modification in pro-inflammatory and anti-inflammatory mediator release.

In vivo studies have also suggested another mechanism for GR activity in that salmeterol and fluticasone can act together to enhance nuclear export of the Th2-specific transcription factor GATA3.[43]

The PKA catalytic subunit can also associate with and phosphorylate GR to modify the ability of GR to repress NF-κB transactivation.[44] This confirms earlier data from Haske et al.[45] Furthermore, PKA has been reported to phosphorylate serine 276 of the p65 subunit of NF-κB directly, thus enabling NF-κB–GR cross talk to occur.[44]

EFFECT OF GLUCOCORTICOIDS ON β_2-RECEPTORS

Up-regulation of receptor number

Dexamethasone, a synthetic glucocorticoid, increases the number of β_2ARs in human lung measured by radioligand binding.[46] Several putative GREs have been identified in the promoter sequence of the human β_2AR gene,[47] and increased β_2AR gene transcription occurs after dexamethasone treatment through a GRE in the 5´-flanking region of the gene[36] in human lung tissue. This increase in transcription is both time-dependent and dose-dependent, consistent with the later induction of receptor binding activity.[36] The mRNA half-life and stability are tissue-specific and cell-specific and are determined to some extent by the level of ribonuclease activity in the cytoplasm of each particular cell type. However, dexamethasone has not been found to alter the half-life of β_2-AR mRNA.[36]

The efficiency of coupling between the β_2-AR and G_s (the G protein that mediates stimulation of adenylyl cyclase) has been reported to be modulated by glucocorticoids.[48] As a result, β_2AR–stimulated adenylyl cyclase activity and cAMP accumulation increase after glucocorticoid treatment. Animals that have been depleted of glucocorticoids by adrenalectomy, in contrast, lose the ability to maintain the sensitivity of the β_2AR–coupled adenylyl cyclase system.[49]

Inhibition of downregulation

Long-term administration of β_2-agonists in vivo causes a marked down-regulation of β_2AR, as measured by mRNA and ligand binding, in human and rat lung. This occurs in a cell-specific manner, with less effect in airway smooth muscle than in lung parenchyma.[50] The agonist-promoted down-regulation of β_2AR may be reversed by treatment with glucocorticoids.[48] Thus glucocorticoids induce an increase in the synthesis of β_2AR in human lung, neutrophils, and lymphocytes.[46] Autoradiographic mapping studies in rats indicate that glucocorticoids upregulate β_2AR and prevent down-regulation of β_2AR in all cell types, including airway smooth muscle cells.[48] Such an effect may have clinical implications for preventing the development of tolerance to β_2-agonists in patients with asthma.

Long-term agonist therapy in patients with asthma results in reduction in β_2AR density in circulating polymorphonuclear leukocytes and lymphocytes,[50] and the down-regulated β_2AR number is restored after administration of oral prednisone. However, a difference in susceptibility to down-regulation between lung and lymphoid tissue may occur.[3]

CONCLUSIONS

LABAs may affect GR nuclear localization through modulation of GR phosphorylation and, further, may prime GR functions within the nucleus by modifying GR or GR-associated protein phosphorylation. Glucocorticoids may in turn regulate β_2AR function by increasing expression, acting through GREs, and, importantly, by restoring G-protein–β_2AR coupling and inhibiting β_2-AR down-regulation, thereby preventing desensitization.

DISCUSSION SESSION

Question: Should salmeterol, formoterol, or short-acting β-agonists have more anti-inflammatory effects in vivo in the presence of endogenous glucocorticoids?
Dr Adcock: Ex vivo data from Omar suggest that in sensitive patients there is an added effect. It is difficult to determine whether this would actually occur in vivo.
Question: What amount of time is required to prime the event to observe translocation?
Dr Adcock: In vivo we see translocation clearly in 30 minutes. Drugs were inhaled at the same time, so it is difficult to determine whether priming occurred. If we look in vitro, depending on the long-acting β-agonist used and the cell type, we can see induction within 30 to 60 minutes. Functionally, there is some effect observed on NF-κB. This may occur through at least two mechanisms. As reported by Evans and Bloom, salmeterol decreases TNFα-stimulated NFκB function and also has an additional effect on the ability of fluticasone to suppress NFκB activity, at least in epithelial cells. This probably occurs independently of DNA binding. Therefore, salmeterol may be enhancing an effect of low levels of endogenous steroid to suppress NFκB activity; an effect that is more pronounced with the addition of exogenous steroid. Alternatively, salmeterol may suppress NFκB function independently of GR.
Comment: One of the effects that has not been reported on yet is the interaction of corticosteroids, β-agonists, and the ability of cells to transmigrate. One of the reasons that we see fewer inflammatory cells after some treatment with the combination of β-agonists and inhaled steroids could be that the cells are being prevented from arriving, not just being made apoptotic or killed. It may also explain why in more severe cases, with patients who seem to be refractory to treatment with the usual drugs, systemic corticosteroids may actually reach the inflammatory cells before they arrive at the airways.
Question: The clinical studies of the superiority of the combinations to the individual agents can be seen for as long as 1 year. Your study of sputum induction of the GR nuclear translocation was an acute study. Have you done any sputum induction studies in chronic dosing, and are these changes still present?
Dr Adcock: We have not performed studies of long-term dosing.
Question: How specific are your results for a given β-agonist?
Dr Adcock: We get the same data in vitro with formoterol and salmeterol. The time course for growth rate translocation differs between cell types and between drugs, which may relate to agonist efficiency. We have not looked at short-acting β-agonists in that regard.
Question: Are there differences seen with GR-α and GR-β?
Dr Adcock: The antibody we used to look for translocation detects

both α and β, so it is difficult to say whether there is a difference between GR-α and GR-β. However, doing Western blots on these with the GR-β–specific antibody, we really do not see anything. With the GR-αβ–specific antibody, we quite often see two bands; it is difficult to know whether one is GR-α and one is GR-β. In peripheral blood cells, we do not detect β, but I think that is an antibody problem. In neutrophils, we see a much higher expression of GR-β at the message than we do any other cell type level. In the sputum cells, we get a nice time-dependent induction of GR nuclear translocation, but it is delayed relative to any other cell type. We do not start to see GR translocation until 2 hours. So whether that has anything to do with how much GR in total there is or there is a GR-β effect, I don't know. Interestingly, Donald Leung has recently reported that GR-β can prevent GR nuclear translocation. One of the reasons why GR work is so difficult to do is that there are not many good antibodies for immunoprecipitation in man. In addition, large numbers of cells are required.

Question: It was previously thought that the interaction between inhaled steroids and β-agonists was a negative one. Might this be a dose-related phenomenon that can be superseded?

Dr Adcock: The initial view that the combination of inhaled steroids and β-agonists is detrimental came from studies involving overexpression of CREB or CBP and the effect on GR functions. It is clear now that there is not a limiting effect of CBP or CREB on many of these functions. In fact, CBP probably acts more as a scaffolding protein than as a functional protein as such. So I think that some of the ideas we had about the molecular functions of these particular cofactors are now altered.

Question: The data that you presented showed that inflammatory cells and cells taken from the sputum demonstrate translocation of the receptor. How does this compare with normal cells from the peripheral blood of a nonasthmatic volunteer?

Dr Adcock: We see the effect even in peripheral blood. We took peripheral blood cells from different groups of patients with asthma of different disease severities, exposed them to dexamethasone with or without 10^{-6} mol/L formoterol. The dexamethasone was labeled to look for GR translocation. Ex vivo we noted a large and additional effect of formoterol on the GR nuclear translocation within 4 hours. We have not studied this in vivo. It may suggest that in some of those patients in whom we see less GR nuclear translocation, both at baseline and after stimulation, that effect could be enhanced and maybe restore some storage function. Kittipong Maneechotesuwan has also shown GR nuclear translocation to occur in peripheral blood cells of normal subjects 60 to 120 minutes after inhaled BOP (800 μg).

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