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Regulation of Chemokine Gene Transcription by Novel Glucocorticoid Receptor Modulators

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Introduction

Diabetes mellitus is a disease state of chronic hyperglycemia caused by insulin resistance and decreased insulin secretion. It is characterized by a loss in pancreatic islet β-cell mass and function (Doria, Patti et al. 2008). As of 2011, 347 million people worldwide suffered from diabetes mellitus (Danaei, Finucane et al. 2011). In the United States, the disease is the seventh leading cause of death, and it increases the risk of other health complications, including cardiovascular disease, stroke, neuropathy, retinopathy, and kidney failure (Morrish, Wang et al. 2001; Alwan, Armstrong et al. 2011; CDC 2011; WHO 2012).

There are two main types of diabetes. Type 2 diabetes, termed adult onset or non-insulin-dependent diabetes, comprises the vast majority of cases of diabetes (WHO 1999; Kriketos, Greenfield et al. 2004). Due to a combination of genetic factors, obesity, and a sedentary lifestyle, type 2 diabetes results in progressive β-cell failure, which leads to a decline in β-cell mass and function and results in deficient insulin secretion and increasing insulin resistance (Kriketos, Greenfield et al. 2004; Alexandraki, Piperi et al. 2006). This loss of β-cell viability is thought to be exacerbated by a cytokine-related systemic inflammation, which has previously been linked to the development of type 1 diabetes (Alexandraki, Piperi et al. 2006).

Type 1 diabetes, formerly called juvenile onset or insulin-dependent diabetes, comprises the minority of diabetes cases and is mostly diagnosed in individuals younger than 18 years of age (WHO 1999; Kriketos, Greenfield et al. 2004). It is characterized by absolute insulin deficiency due to autoimmune-mediated selective destruction of insulin-producing β-cells (Kriketos, Greenfield et al. 2004; Eizirik, Colli et al. 2009). This autoimmune-induced diabetes is thought to be associated with the release of chemotactic cytokines, or chemokines. Cytokines are a group of pharmacologically active proteins that affect the inflammatory and immune systems by autocrine and paracrine modes of action, and chemokines are a family of small cytokines that induce leukocyte migration to sites of injury via cellular chemotaxis (Fernandez and Lolis 2002; Alexandraki, Piperi et al. 2006). Cytokines and chemokines, especially those secreted from adipose tissue, trigger an immune cell invasion of pancreatic islets via inflammatory signaling pathways (Alexandraki, Piperi et al. 2006; Deshmane, Kremlev et al. 2009).

One such chemokine involved in this process is monocyte chemoattractant protein-1, or CCL2. CCL2 is responsible for the recruitment of leukocytes, monocytes, macrophages, and T-cells to islet tissues and the subsequent release of toxic pro-inflammatory cytokines, such as IL-1β (Hayden and Ghosh 2008; Deshmane, Kremlev et al. 2009; Oeckinghaus, Hayden et al.
IL-1β is an inflammatory, cytotoxic cytokine that decreases β-cell function and viability. Along with IL-6, IL-1β increases the risk of developing diabetes by inhibiting β-cell function and inducing β-cell death via autoimmune destruction (Alexandraki, Piperi et al. 2006).

The increased expression of pro-inflammatory cytokines in peripheral blood mononuclear cells occurs with an increase in pro-inflammatory genes transcription regulated by nuclear factor-κB, or NF-κB (Alexandraki, Piperi et al. 2006). In non-stimulated cells, the latent dimer form of NF-κB and its subunits, including p65 and p50, are bound to IκB, an NF-κB inhibitor, and its corresponding subunits (Devaraj, Dasu et al. 2010). Upon stimulation by cytokines, IκBα is phosphorylated and effectively degraded, allowing NF-κB subunits, such as p65 (Rel A) and p50, to accumulate in the nucleus. These major protein components of NF-κB affect gene expression by binding κB elements in specific gene promoters and increasing the synthesis and secretion of monocyte chemokines and cytokines (Alexandraki, Piperi et al. 2006; Devaraj, Dasu et al. 2010). Through this NF-κB pro-inflammatory signaling pathway, cytokines are able to regulate the transcription of a number of genes related to β-cell death, including that of CCL2 (Hayden and Ghosh 2008; Deekinghaus, Hayden et al. 2011). The over-expression of these pro-inflammatory cytokines results in an increase in β-cell impairment and destruction, insulitis, and eventual diabetes (Alexandraki, Piperi et al. 2006; Martin, Rankin et al. 2008; Ogliari, Caldara et al. 2008).

While the inflammatory signaling pathways regulating CCL2 expression in pancreatic islets are not completely understood, therapeutic drugs with anti-inflammatory actions are frequently used in diabetes to decrease inflammation (Biju, McCormick et al. 2011). Among these are glucocorticoids, a class of catabolic steroid hormones known to regulate glucose synthesis and metabolism (Netter 1965). Synthesis and release of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal axis, and serum levels vary by circadian rhythm and elevate in response to physical and psychological stresses (Clark and Belvisi 2012). Glucocorticoids work through both genomic and non-genomic mechanisms to suppress pro-inflammatory pathways and promote anti-inflammatory pathways. Through binding action with the glucocorticoid receptor (GR), glucocorticoids can form a GR dimer that uncovers a zinc finger DNA-binding site attached to a glucocorticoid response element (GRE), regulating specific target genes and suppressing the NF-κB pathway (Rhen and Cidlowski 2005; Witchel and DeFranco 2006; De Bosscher and Haegeman 2009). The receptor can also interact with coregulator proteins, controlling transcriptional activation or repression without direct interaction with DNA (Witchel and DeFranco 2006). The coordinated regulation of a diverse number of
target genes within the cell promotes an anti-inflammatory state that offsets the toxic effects of pro-inflammatory pathways (Witchel and DeFranco 2006; De Bosscher and Haegeman 2009).

The most abundant naturally-occurring glucocorticoids are the adrenocortical hormones cortisol and cortisone (Netter 1965; Davis 1981). Cortisol is the active form of cortisone, and it plays an important regulatory role in the metabolism of macronutrients and electrolytes (Davis 1981). Derived from cholesterol, both steroid hormones are synthesized principally in the zona fasciculata of the adrenal cortex, though synthesis also occurs in areas such as the primary lymphoid organs, intestines, skin, and central nervous system (Clark and Belvisi 2012). Cortisol is referred to pharmaceutically as hydrocortisone and is commonly used as an anti-inflammatory agent (Davis 1981).

Hydrocortisone-derived synthetic glucocorticoids can possess improved glucocorticoid receptor affinity, nuclear receptor selectivity, or an increased duration of action as compared to endogenous hydrocortisone, increasing their anti-inflammatory efficacy (Biju, McCormick et al. 2011; Gilman, Goodman et al. 1980). Figure 1 shows the basic structure of cortisol and the points of modification determining the characteristics of synthetic compounds. Structures necessary for the characteristic anti-inflammatory and carbohydrate-regulating effects of adrenocorticosteroids include: the 4,5 double bond, the 3-ketone, and the 11-hydroxyl group (Gilman, Goodman et al. 1980). Points of modification, shown in bold in Figure 1, can yield a variety of compounds with different characteristics. For example, the addition of a 1,2 double bond, seen in prednisolone and prednisone derivatives of cortisone and cortisol respectively, improves anti-inflammatory effects and enhances the ratio of carbohydrate-regulating potency to sodium-retaining potency (Gilman, Goodman et al. 1980). The 17α-hydroxy group is the main differentiator between cortisol and corticosterone, and it lends to the full expression of anti-inflammatory and carbohydrate-regulating effects of cortisol. Finally, the 21-hydroxy group, found in all natural corticosteroids and most synthetic forms, is required for significant sodium-retaining activity and some glycogenic and anti-inflammatory activities (Gilman, Goodman et al. 1980).

Figure 1: Structure-function relationship in adrenocorticosteroids. Bold structures indicate modification points for synthetic derivatives. Source: Gilman 1980
Among the most commonly used synthetically-derived glucocorticoids are prednisolone, dexamethasone, and fluticasone propionate (Biju, McCormick et al. 2011). Prednisolone, shown in Figure 2, has increased anti-inflammatory efficacy as well as a slower relative metabolism compared to cortisol (Gilman, Goodman et al. 1980). Dexamethasone, or 9α-Fluoro-16α-methyl prednisolone, is shown in Figure 3. With no sodium-retaining potency, dexamethasone has a long duration of action and a much greater relative anti-inflammatory effect, requiring a much lower equivalent dose as compared to cortisol (Gilman, Goodman et al. 1980). This potent synthetic glucocorticoid is used to suppress endogenous release of cortisol and adrenocorticotropic hormone and is used to treat a variety of inflammatory-based disorders ranging from acute injury to organ function to certain types of cancer (Nussey and Whitehead 2001; Petrella, Ercolino et al. 2006; Choi, Jo et al. 2013). Fluticasone propionate, or S-(fluoromethyl)6α,9-difluoro-11β-17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate,17-propionate, is shown in Figure 4. Fluticasone propionate possesses strong anti-inflammatory potency, displays activity similar to progesterone, and has shown a glucocorticoid receptor binding and gene expression three to five times more potent than dexamethasone (Smith 2011). Fluticasone propionate also shows some affinity for progesterone receptor binding (Issar, Sahasranaman et al. 2006). It has wide therapeutic use in nasal sprays to alleviate allergy symptoms (Smith 2011).
Despite the anti-inflammatory benefits of glucocorticoids, a number of systemic side effects can occur from overdosage or prolonged use (Schacke, Docke et al. 2002). In response to overexposure, the glucocorticoid receptor can experience a hormone-dependent down-regulation, which can alter stress responses and reduce inflammatory response. While this can be beneficial on a cellular level as a feedback mechanism, the change in hormonal response can have negative consequences on an organ system level (Witchel and DeFranco 2006). Chronic use of glucocorticoids can disrupt hypothalamic-pituitary-adrenal axis homeostasis and suppress its function, causing a wide range of adverse effects on metabolism, the cardiovascular system, and within the central nervous system (Clark and Belvisi 2012; Nam, Kim et al. 2013). Side effects from glucocorticoid use include increased susceptibility to infection, hypertension, neurocognitive impairment, hyperglycemia, muscle atrophy, osteoporosis, and impaired carbohydrate homeostasis (Gilman, Goodman et al. 1980; Witchel and DeFranco 2006; Clark and Belvisi 2012). Over-exposure to glucocorticoids can inhibit glucose-stimulated insulin secretion in the pancreas and peripheral glucose uptake in the muscles, leading to immunosuppression, β-cell dysfunction, and impaired glucose tolerance (Witchel and DeFranco 2006; Nikolic, Vujicic et al. 2013).

Compromised insulin-stimulated glucose uptake in the muscles seen in insulin resistance is characteristic of metabolic syndrome and Cushing’s syndrome (Witchel and DeFranco 2006). Metabolic syndrome, also known as insulin resistance syndrome or syndrome X, is a disorder of chronic pro-inflammatory activation characterized by a group of risk factors including central obesity, hyperglycemia, hypertension, and dyslipidemia (Witchel and DeFranco 2006; A.D.A.M. Medical Encyclopedia 2012). While central obesity and insulin resistance are the main risk factors, metabolic syndrome is also exacerbated by aging, hormonal changes, genetic predisposition, sedentary lifestyle, and systemic inflammation (A.D.A.M. Medical Encyclopedia 2012). Metabolic syndrome increases the risk of cardiovascular disease, stroke, and type 2 diabetes, and it is becoming increasingly problematic in the United States (Witchel and DeFranco 2006; A.D.A.M. Medical Encyclopedia 2012).

Cushing’s syndrome is a hormonal disorder characterized by chronic exposure to excessive glucocorticoid levels (Witchel and DeFranco 2006; Prague, May et al. 2013). While hypercortisolemia can be due to endogenously rooted hormonal imbalances, Cushing’s syndrome is more commonly caused by prolonged use of glucocorticoid therapeutic drugs. Slowly progressive and non-specific in symptoms, Cushing’s syndrome patients may suffer from a combination of weight gain, depression, skeletomuscular weakness, and headaches. Cushing’s syndrome is linked to diabetes and hypertension and can cause myopathy, congestive cardiac
failure, reproductive disfunction, osteoporosis, and mood disturbance. Untreated Cushing’s syndrome is linked with 50% mortality within five years (Prague, May et al. 2013).

Cushing’s syndrome represents an archetype of metabolic syndrome, as the majority of signs and symptoms are shared between the two, and effective treatment of Cushing’s syndrome would also improve the main symptoms of metabolic syndrome (Chanson and Salenave 2010). Decreasing intracellular cortisol levels is shown to improve metabolic status. Therefore, it is important to understand glucocorticoid-based β-cell disfunction, as well as improve upon the tissue-selective action of glucocorticoids through synthesis of novel compounds that can mimic the beneficial actions of cortisol without the undesirable side effects (Witchel and DeFranco 2006).

The transrepression of the pro-inflammatory NF-κB pathway is thought to be the major anti-inflammatory effect of glucocorticoids. The role of transactivation of genes by glucocorticoids, however, is disputed (Biju, McCormick et al. 2011; Clark and Belvisi 2012). Some studies have suggested that the majority of side effects seen in glucocorticoid use are due to transactivation via GR receptor-steroid dimer complex interaction with DNA. The transactivation properties have long been suggested to be key in determining the metabolic side effects of glucocorticoids (Newton and Holden 2007). However, further studies have shown evidence contrary to this belief, attributing some beneficial anti-inflammatory effects to glucocorticoid gene transactivation as well as transrepression (Burke, Goff et al. 2012; Clark and Belvisi 2012). The newer studies argue that the varied and interdependent actions of glucocorticoids cannot be neatly divided into separate results of transactivation versus transrepression. In addition, glucocorticoid actions seen as undesirable side effects when over-expressed are evolutionarily advantageous within the feedback system of endogenous glucocorticoids, further complicating any distinctions that may be drawn about a subset of glucocorticoid actions (Clark and Belvisi 2012).

Nevertheless, efforts continue to be made to synthesize a glucocorticoid with dissociative properties, or one that can separate transactivation and transpression activities, in hopes of creating a potential therapeutic drug with improved safety profile to treat inflammatory-linked diseases (Biju, McCormick et al. 2011). Despite controversy surrounding dissociative modes of action, the synthesis of novel compounds can still yield glucocorticoids with improved anti-inflammatory function with minimized side effects due to slight changes in transcriptional regulatory activity or altered pharmacological properties (Clark and Belvisi 2012).
Previous synthetic hydrocortisone derivatives, while effective at mimicking and improving upon the beneficial anti-inflammatory functions of the endogenous hormone, also carry with them the associated negative side effects. For instance, dexamethasone has shown in vivo to induce hyperinsulinemia and insulin resistance in a dose-dependent manner without notable hyperglycemia (Rafacho, Cestari et al. 2009).

Recently, C-21 mercapto-benzothiazole compounds (mercaptosteroids) have been prepared from hydrocortisone through a process described in Figure 5 (Biju, McCormick et al. 2011). In particular, mercaptosteroids 4 and 6 from this synthesis have shown improved safety profiles, with mercaptosteroid 4 also demonstrating a promising dissociative profile. Mercaptosteroid 4 has shown moderate inhibition of IL-6 cytokine synthesis and significant inhibition of inflammatory cell influx as compared to fluticasone propionate. Along with significant in vivo anti-inflammatory activity, it has demonstrated a better safety profile. Studies both in vitro and in vivo focused on the therapeutic potential of mercaptosteroid 6 in asthma showed effective lung function preservation and suppression of pulmonary inflammation with an improved safety profile as compared to fluticasone propionate (Biju, McCormick et al. 2011).

Due to the particularly promising results of C-21 mercapto-benzothiazole compound (mercaptosteroid) 4 in its anti-inflammatory use within the lungs, this study seeks to examine the anti-inflammatory action of this compound within the pancreatic β-cell. It is hypothesized that this compound will suppress the pro-inflammatory action of the CCL2 gene without impairing β-cell function, proving to be a potential therapeutic intervention for β-cell-related disorders.
Methods

The cell line used in these experiments was 832/13, a rat insulinoma (INS-1) derived cell line containing the human proinsulin gene that shows a more sensitive glucose response (Hohmeier, Mulder et al. 2000). Plasmid vectors containing a luciferase reporter gene were inserted into the cells. To measure transactivation, glucocorticoid response element (3XGRE) was the reporter gene driving luciferase. For transpression, CCL2 reporter gene drove luciferase. The compounds hydrocortisone and mercaptosteroid 4 used in this research were generated in Dr. Shawn Campagna’s organic chemistry laboratory at the University of Tennessee Department of Chemistry. Compounds were each diluted from powder into dimethyl sulfoxide (DMSO), which were then diluted into media for application into cell culture. Dilution factors used were 10µmol (-5), 1µmol (-6), 0.1µmol (-7), 10nmol (-8), 1nmol (-9), and 0.1nmol (-10).

Once cells were harvested, luciferase assays were performed according to the manufacturer’s instructions (Promega). Following luciferase assays, bicinchoninic acid (BCA) assays were performed to normalize protein content of each well plate to total protein content. DMSO served as the vehicle control. The control IL-1β-mediated CCL2 promoter activity was set at 100%, and the control transactivation of synthetic glucocorticoid responsive promoter termed the 3XGRE was set at 1.0. Transrepression of IL-1β-mediated CCL2 promoter activity was measured as percent reduction in expression, while transactivation of 3XGRE was analyzed as fold over control. The resulting transactivation and transpression due to mercaptosteroid 4 were compared to that of hydrocortisone.

Results

As expected, decreased IL-1β induction of CCL2 was observed in the presence of hydrocortisone. For the hydrocortisone doses ranging form 0.1nmol (-10) through 1µmol (-6), repression of IL-1β-mediated CCL2 promoter activity was approximately 50% to 80% as compared to the control. For dose 10µmol (-5) IL-1β response was approximately 25% of the control. Mercaptosteroid 4 displayed similar transrepression capabilities. Relative to the control promoter activity, mercaptosteroid 4 exhibited dose-dependent repression of IL-1β induction of CCL2 reporter gene. By dose 1µmol (-6), IL-1β expression was less than 50% compared to the control. At dose 10µmol (-5), IL-1β response was approximately 25% of the control.

In contrast, transactivation of synthetic glucocorticoid responsive promoter 3XGRE was observed in hydrocortisone but not with exposure to mercaptosteroid 4. With hydrocortisone, no
significant increase in expression over control was observed in doses 0.1nmol (-10) or 1nmol (-9). Expression increased 4-fold over control by dose 10nmol (-8), rose approximately 8-fold over control with doses 0.1µmol (-7) and 1µmol (-6), and showed transactivation of approximately 10-fold over control with dose 10µmol (-5). Transactivation due to mercaptosteroid 4 was not observed with doses 0.1nmol (-10) through 1µmol (-6). At dose 10µmol (-5), transactivation over control increased to nearly 2-fold over control. This increase in expression is still less than was observed with hydrocortisone at a lower dose of 10nmol (-8).

Discussion

Based on the observed results, mercaptosteroid 4 appears as effective as hydrocortisone at transpressing IL-1β-mediated CCL2 promoter activity. This indicates that mercaptosteroid 4 may effectively inhibit toxic pro-inflammatory pathways in the pancreas, as CCL2 and IL-1β are linked with pancreatic β-cell destruction via inflammatory responses (Alexandraki, Piperi et al. 2006; Hayden and Ghosh 2008; Deshmane, Kremlev et al. 2009). While the specific significance
of the presence of transactivation is still debated, the lack of transactivation of synthetic glucocorticoid responsive promoter 3XGRE from mercaptosteroid 4 as compared to hydrocortisone may be an indication that transactivation-induced side effects may not be present.

Overall, results suggest that mercaptosteroid 4 could have promising potential as a therapeutic agent similar to hydrocortisone in type 1 diabetes mellitus. Further studies are needed to understand the impact of the dissociative properties of mercaptosteroid 4. Alternative experiments aimed at measuring undesirable side effects through other mechanisms could also be performed. Finally, similar studies discussed in this paper examining transrepression and transactivation could also be performed using mercaptosteroid 6.

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