Protein phosphatase 5 and glucocorticoid receptor β in glucocorticoid resistance and lipogenesis

Terry D. Hinds
Medical University of Ohio

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A Dissertation

Protein Phosphatase 5 and Glucocorticoid Receptor β in

Glucocorticoid Resistance and Lipogenesis

by

Terry D. Hinds, Jr.

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Medical Science

_______________________________________
Dr. Edwin R. Sanchez, Committee Chair

_______________________________________
Dr. Sonia Najjar, Committee Member

_______________________________________
Dr. Kam Yeung, Committee Member

_______________________________________
Dr. Ronald Mellgren, Committee Member

_______________________________________
Dr. Xiaodong Wang, Committee Member

_______________________________________
Dr. Patricia Komuniecki,
Dean of the College of Graduate Studies

The University of Toledo
December, 2010
An Abstract of
Protein Phosphatase 5 and Glucocorticoid Receptor β in Glucocorticoid Resistance and Obesity

by
Terry D. Hinds, Jr.

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Medical Science

The University of Toledo
October 21, 2010

Glucocorticoid (GR) and peroxisome proliferator-activated receptor γ (PPARγ) play important roles in adipocyte differentiation by controlling the balance between lipolysis and lipogenesis. In this work, we show that the nuclear receptor co-chaperone, protein phosphatase 5 (PP5), and GR isoform, GRβ, are key regulators of adipogenesis. Glucocorticoids (GC) are well-known antagonists of insulin and long-term GC treatment results in insulin resistance and obesity, however, acute treatment results in lipolysis and weight loss. These GC effects are mediated by the GR, specifically the GRα isoform. In this dissertation, we show that PP5 and GRβ are inhibitory to the actions of the hormone-binding isoform, GRα. Loss of PP5 elevated dexamethasone (Dex)-induced GRα activity at reporter and pro-lipolytic endogenous genes, such as PDK4 that correlated with increased phosphorylation of GR. GRβ exhibits a degenerate amino acid sequence in helix 12 that abrogates ligand-binding and acts as a dominant-negative inhibitor of hGRα.
We also demonstrate hormonal and dietary control of mGRβ expression. In vitro, upregulation of mGRβ was observed in response to dexamethasone, inflammatory cytokine TNFα and insulin. In vivo studies to confirm the involvement of mGRβ in metabolism showed significantly elevated levels of mGRβ in the livers of mice subjected to fasting-refeeding. Mice on a long-term high-fat diet developed insulin resistance with a significant increase in hepatic and visceral adipose GRβ and TNFα. Interestingly, PP5 only increased in the visceral adipose of these mice, which suggest a possible role of regulation on lipogenesis. Rosiglitazone-induced PPARγ activity in PP5KO MEF cells was greatly reduced at lipogenic genes, such as aP2 and CD36. These data suggest that PP5 serves as a reciprocal modulator by exerting negative control over the lipolytic actions of GRα, while promoting the lipogenic properties of PPARγ.

In mouse 3T3-L1 preadipocyte cells, levels of three proteins known to inhibit GRα activity, GRβ, PP5 and FKBP51, were highly elevated in the differentiated state, while expression of FKBP52 – a positive GR regulator – was unchanged. Importantly, PP5 KO cells were highly resistant to differentiation, showing almost no intracellular lipid accumulation and higher levels of free fatty acids (FFA) in the media. These phenotypes were completely reversed in PP5KO cells following reintroduction of PP5. We propose that obesity is a result of GC-resistance, and that mGRβ or PP5 may work to increase lipid storage by inhibiting the actions of GRα.
Dedication page

Dedicated to the memory of my grandfather, Thomas Elswick (1934-2009).
Acknowledgments

This dissertation would not have been possible with the love, support and encouragement I received from my friends and family. Dr. Edwin Sanchez has been tremendous in guiding and helping me over the years and I am very appreciative of his mentorship. I would like to also thank Drs. Sonia Najjar and Rudel Saunders, as well as, Sadeesh Ramakrishnan and Lance Stechschulte for their friendship, advice and assistance in thinking through ideas.
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Introduction

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Chapter 1

Introduction

Obesity is an ongoing epidemic that needs immediate attention. The development of lean tissues in mammals, that is to reduce the amount of body fat, has been linked to a significant decrease in diseases such as type 2 diabetes mellitus, cardiovascular disease, atherosclerosis and some cancers [1]. Increases in these diseases have been associated with sedentary lifestyle and a diet that is high in fat [1, 2]. Controlling dietary intake and exercise have been shown to give the best results in significantly decreasing body fat percentage [2]. In humans, obesity often results from alterations to carbohydrate and lipid metabolism, processes that are principally controlled by insulin and glucocorticoids (GCs) [3]. In the fasted state and during exercise, GCs stimulate several processes that collectively serve to increase and maintain normal concentrations of glucose in blood [3] [4]. Stimulation of gluconeogenesis, particularly in the liver, results in synthesis of glucose from non-hexose substrates such as amino acids and lipids [3] [4]. As well, GCs stimulate fat breakdown in adipose tissue and inhibit glucose uptake in muscle and adipose tissues [4]. Thus, GCs are best categorized
as physiologic antagonists to insulin, which promotes glucose uptake and storage of lipids (lipogenesis).

Chronic GC elevation leads to insulin resistance and glucose intolerance, which may result in type II diabetes mellitus and possibly the metabolic syndrome, which symptoms are identified by: visceral obesity, high blood pressure, insulin resistance or abnormal cholesterol levels — altogether these may increase risk of heart disease, stroke and diabetes. Prolonged obesity may also result in inflammation of liver and adipose that can lead to non-alcoholic fatty liver disease (NAFLD) and then possibly non-alcoholic steatohepatitis (NASH). It is thought that GCs exacerbate visceral obesity and weight gain because Cushing’s syndrome (hypercortisolism) patients have symptoms similar to the metabolic syndrome, even though the actions of GCs are to work as anti-inflammatories, these patients still can develop inflammation of adipose [5] and liver that may lead to NAFLD [6]. The adipose tissue secretes proinflammatory cytokines, named adipokines, which contribute to adipocyte hypertrophy. Adipokines such as resistin, chemerin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) contribute to insulin resistance and inflammation of the adipose. The actions of proinflammatories and anti-inflammatories appear to be unbalanced in the severely obese, which may contribute to the inflammation and invasion of immune cells into tissues.

Interestingly, the protein receptor for cortisol, the glucocorticoid receptor (GR), was increased in hamsters that performed exercise for 6 weeks, which
resulted in increased lipolysis in both regular and high-fat diets of the young and old [7]. Xu et al. demonstrated in rat primary adipocytes that GR controls lipolysis by suppressing cyclic nucleotide phosphodiesterase 3B, which elevates cellular cAMP production and activating PKA [8]. PKA activates hormone sensitive lipase (HSL) that, in turn results in HSL-perilipin interaction, which leads to phosphorylation of perilipins and catalysis of triglycerides (TG) and diglyceride breakdown, producing a subsequent release of free fatty acids (FFA) and glycerol [8] [9]. The perilipins belong to a family of hydrophobic lipid droplet-associated phosphoproteins that are phosphorylated by PKA in multiple residues [9]. Perilipins are adipose specific proteins that are located on the surface of the lipid droplet and functions to prevent lipolysis of TGs during basal conditions [9]. Perilipin expression is increased by the master fat regulatory gene, peroxisome proliferated activated receptor (PPAR)-γ, to coat lipid droplets in adipocytes that may result in adipocyte hypertrophy [10]. GR has been shown to down-regulate perilipins, fatty acid binding protein - adipocyte Protein 2 (aP2), and fatty acid and glucose scavenger receptor - cluster of differentiation 36 (CD36) expression that are involved in lipogenesis. Additionally, GR upregulates the expression of genes involved in lipolysis, such as HSL, DGAT1, glucocorticoid-induced leucine-zipper (GILZ) [11], serum- and glucocorticoid-inducible kinase (SGK) [12], and angiopoietin-like 4 (ANGPTL4) [13]. We hypothesize that the balance of GR and PPARγ activity may be altered in
adipocytes of the obese to result in adipocyte hypertrophy, and manifestation of GC resistance.

Glucocorticoid Resistant Disease States

Due to underlying pathology or drug treatment, GC resistance can develop and is now a major concern in many disease states. GC resistance is defined as the inability of tissues, or more specifically, the GR, to respond to endogenous glucocorticoid hormones or to potent synthetic agonists. The GR is a hormone-activated transcription factor that controls many physiological processes, extending from glucose metabolism to lung development [2] [1]. Not surprisingly, loss of these actions results in dire consequences, as demonstrated by lethality of GR-ablated mice [14]. There are several steps required for generation and activation of GR and also in regulating GR controlled gene responses. This provides numerous avenues that may alter GR activity either positively or negatively, which can result in GC sensitivity or resistance. As a steroid receptor, the early steps

Figure 1. GR Heteromeric Complexes. See text for description.
required for GR include: generation of hormone-competent receptors through a HSP90-based chaperone system, the hormone-binding event, hyperphosphorylation upon hormone binding, dissociation of the GR complexes, translocation to the nucleus and binding of glucocorticoid response elements (GREs) at gene promoters [1] [15]. Mature forms of the steroid receptor heteromeric complexes contain one molecule of receptor, two molecules of HSP90, and one molecule of a stabilizing protein - p23 (Figure 1) [15]. The HSP90 dimer generates a single binding cleft for one tetratricopeptide repeat (TPR) protein, termed TPR-binding domain (TBD). There are four known TPR proteins that bind to mature GR heteromeric complexes; FK506-binding protein 51 (FKBP51), FK506-binding protein 52 (FKBP52; also known as HSP56), cyclophilin-40 (Cyp40), and protein phosphatase 5 (PP5).

Recently, our lab has demonstrated that when GR is bound to agonist an exchange of FKBP51 for FKBP52 occurs that coincides with translocation of GR to the nucleus [16] [17]. FKBP52 knockout (KO) mouse embryonic fibroblast (MEF) cells have reduced GR regulated gene responses [54], demonstrating the importance of this protein for GR regulated activity. FKBP51 KO cells have increased GR regulated gene responses and it was also shown that knock down of PP5 via siRNA had a similar modulatory effect [18]. Suggesting that both FKBP51 and PP5 are negative regulators of GR and FKBP52 a positive regulator.

In humans, GC resistance has been shown to occur by two major mechanisms: loss-of-function mutations in the major hormone binding isoform,
GRα [19], or by increased expression of GRβ, which does not bind glucocorticoids and acts as a dominant-negative inhibitor of GRα. Although GRα mutations can result in a type of GC resistance that is both systemic and severe, these mutations are rare. In contrast, the evolving evidence suggests that GC resistance based on GRβ or by inhibitory co-chaperone proteins PP5 and FKBP51 are much more common and likely to be tissue-specific in nature. To date, GC resistance, via GRβ appears to occur in many immunological diseases and drug-resistant states, such as asthma, leukemia, ulcerative colitis, chronic sinusitis, and systemic lupus erythematosus. Patients suffering from these diseases can be refractory to GC treatment. Not surprisingly, increased activation of pro-inflammatory factors and elevated hGRβ expression has been noted in cases of GC resistance.

Hypercortisol secretion leads to symptoms similar to the metabolic syndrome, but the actions of GR in these patients may not be highly active. A study by Huizenga et al. showed that Cushing’s syndrome patients had no difference in the mean number of GR proteins, but did have significantly lower hormone binding affinity to the receptors [20]. The diminished ligand binding affinity leans toward the development of GC resistance in people with high cortisol. A later study showed that this difference was by increased expression of GRβ, and no change in GRα [21]. These observations point to the emerging importance of hGRβ as a homeostatic mechanism in the normal attenuation of
GC responses and as a cause in hormone-resistant disease states. Surprisingly, GC resistance has not yet been demonstrated in diseases of metabolism.

GRβ, FKBP51 and PP5 provide many avenues of GC resistance, and could be tissue specific in nature because these proteins are expressed at different levels in tissues throughout the body. GR sensitivity occurs when FKBP51 or PP5 are suppressed or knocked out. Furthermore, local amplification of cortisol can occur by an enzyme, 11β hydroxysteroid dehydrogenase type 1 (11β HSD1) that can convert cortisone (the non-GR binding form) to cortisol (binds and activates GR), and this may result in high levels of cortisol within a tissue and exacerbate GR activity.

Glucocorticoids and 11β HSD1 and Their Roles in Obesity

Adipose tissue allows storage of fatty acids and triacylglycerol in periods of energy excess and the consequent use of triacylglycerol stores during energy deprivation. Possible causes of obesity are twofold: 1) an increase in the adipose cell precursor population, or 2) alteration of the lipogenic/lipolytic equilibrium that results in adipocyte hypertrophy [2] [22] [23]. Excess GCs (cortisol in humans and corticosterone in rodents) causes metabolic syndrome-like phenotype, such that is seen in patients with Cushing’s syndrome. However, cortisol levels are normal in obese patients [24]. Interestingly, acute GC treatment results in lipolysis of triglycerides in adipocytes and weight loss, on the other hand, chronic GC exposure results in weight gain [25]. We are interested in the
difference of acute versus chronic treatment and why GC’s have an opposite effect on the same tissue.

It has been well documented that differentiation from early preadipocytes can be performed in culture with a cocktail of insulin, isobutylmethylxanthine (IBMX), and GC treatment (DMI cocktail) (Figure 2) [26]. The induction conditions and media vary according to the cell lines. Treatment of cells with IBMX activates the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) [26] [27]. IBMX inhibits soluble cyclic nucleotide phosphodiesterases and results in increase intracellular cAMP levels. Insulin promotes adipocyte differentiation by activating PI3-kinase and Akt activity. Modulation of the activity of the forkhead transcription factor Foxo1 appears to be necessary for insulin to promote adipocyte differentiation [26]. At the nuclear level, treatment with GCs results in activation of the related transcription factor C/EBPδ [26]. C/EBPβ and δ, in turn induce transcription of C/EBPβ and PPARγ [26]. Thus, C/EBPβ and PPARγ direct the final phase of adipogenesis by activating

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**Figure 2. Adipogenesis Model.** The DMI cocktail induces preadipocytes cells to differentiate into adipocytes.
adipocyte-specific genes, mostly those involved in de novo fatty acid synthesis and lipogenesis, such as perilipins, fatty acid synthetase, lipoprotein lipase (LPL), sterol regulatory binding protein-1c (SREBP-1c) and liver X receptor α (LXRα) [26] [28].

The enzyme, 11β HSD1 is upregulated on a high fat diet and after adipose differentiation of 3T3-L1 preadipocyte cells, and this may sensitize these cells to GC treatment [29]. A transgenic mouse model that had 11β HSD1 overexpressed in adipose tissue, via aP2 promoter, showed significant weight gain and development of visceral adiposity that was shown to be related to increased adipocyte size, and not adipocyte number [30]. The adipocyte hypertrophy and weight gain was correlated to actions of GR, even though GR activity was not tested. Interestingly, knockdown of total GR in 3T3-L1 cells resulted in no change in the ability of the adipocytes to differentiate compared to siRNA control [31]. The mineralcorticoid receptor (MR), however, showed to impact adipocyte differentiation when knocked down and resulted in significantly less lipids compared to controls [31]. The study also went on to show that under hormone-free conditions adipocyte differentiation could occur with aldosterone treatment [31], and that dexamethasone suppresses adipogenic genes adiponectin, leptin, resistin and aP2, which demonstrates an inhibition by GR and induction by MR in adipocytes.

Knockout of 11β HSD1 in mice had no significant difference in weight gain compared to wild type, but did have lower blood glucose on glucose
tolerance test (GTT) [32]. The 11β HSD1 KO also had significantly more plasma corticosterone production and increased adrenal weights, as well as decreased adipokines. GCs are well known to suppress cytokines and adipokines, which could mean a more active GR in the KO, because of the increased corticosterone production. Inactivation of cortisol is regulated by 11β HSD2 that reverses cortisol to non-GR binding cortisone, that still binds MR. Overexpression of 11β HSD2 via the aP2 promoter and challenge with a high fat diet resulted in transgenic mice that was resistant to diet induced obesity [33]. The transgenic mice did have decreased food intake, increased energy expenditure and improved glucose tolerance and insulin sensitivity. They also had lower expression of leptin, which may have influenced weight loss and dietary intake, and may be the sole reason for the weight loss. Interestingly, the 11β HSD2 knockout mice are markedly hypertensive. A mutation in 11β HSD2 gene that results in loss of activity has been demonstrated in humans that results in the syndrome of apparent mineralocorticoid excess (SAME), which these patients have hypertension and hypokalemia. Interestingly, a study by Mussig et
al. showed that in severe obese humans there were no difference in activity of 11β HSD1 and 5α reductase, but there was a significant increase in activity of 11β HSD2 [24], which would demonstrate an inhibition of GR and possibly an increase of MR activity, even though the 11β HSD2 knockin mice did not demonstrate this phenomena.

Unlike 11β HSD2, 11β HSD1 requires hexose-6-phosphate dehydrogenase (H6PDH) for the conversion of cortisone to cortisol (Figure 3); otherwise the same enzyme can convert cholesterol to oxyersterols that are LXRα agonist [34]. LXRα is well known to induce lipogenesis and adipocyte hypertrophy [35] [36]. Thus, GR may not be causing this phenotype and it may be GC resistance and reduced lipolysis. 11β HSD1 is an important enzyme, even if GR is not the culprit causing adipocyte hypertrophy, as was shown with 11β HSD1 inhibitor, carbenoxolone, which can reduce plasma insulin, glucose and total cholesterol [37]. Analysis of the GR gene shows two isoforms, GRα and β. The GRβ isoform has been shown to be increased by GCs and may be a player in GC resistance from long-term exposure or treatment that may result in obesity.

**Structure and Expression of the Glucocorticoid Receptor Gene**

Sequencing of human GR (hGR) cDNAs in 1985 unveiled two GR isoforms, α and β (Figure 4) [38]. These isoforms differ in their poly-A tails and additional studies revealed that they result from alternative splicing of a single GR gene. Translations of the two transcripts results in a GR that can bind to GCs
(GRα), and a GR that cannot (GRβ). The following year, mouse GR (mGR) cDNAs also revealed a single GR gene with only one GR protein that would respond to glucocorticoids. However, like human GR, two different mRNAs were found with distinct poly-A tails [39] [40]. Interestingly, of the two mRNA transcripts from mGR, only one was pursued, GRα, and this has been the most extensively studied isoform. The GR protein-coding region is produced by exons 2–9, where exon 1 represents the 5’-untranslated region with multiple promoters and transcription-initiation sites that regulate the expression of GR [41] [42]. Both humans and rodents produce multiple GRα isoforms (A–D) as a result of an alternative usage of subregions within exon 1 and fusion to a common exon 2 after splicing. Thus, humans and rodents express several isoforms of GRα that
vary in the N-terminus but which have the same hormone-binding region. In humans, alternative splicing has also been documented in the distal region of the GR genomic sequence, such that alternative usage of splice acceptor sites in exon 9 results in production of hGRα and hGRβ mRNA [43]. Splicing of exon 9 adds 50 amino acids to exon 8 to produce the activation factor 2 (AF2) of the ligand binding domain. The hGRβ adds 15 amino acids to exon 8 that results in a loss of AF2 and ligand binding function. As a result, GRβ is inhibitory to the GRα isoform by protein-protein interactions [44] and by recruiting histone deacetylases (HDACs) to gene promoters that would have normally been activated by GRα (Figure 5) [45].

Figure 5. Alternative splicing of mGR gene to produce α and β isoforms. Mature GR heteromeric complexes bind HSP90 and TPR proteins. FKBP51 and PP5 are inhibitory to the actions of GR, and FKBP52 a positive regulator. GRα isoforms homodimerize on gene promoters to activate or suppress GR regulated genes. GRβ heterodimerizes with GR α on gene promoters and by protein-protein interactions that are inhibitory. PDK4 is an anti-lipogenic gene that GRβ was shown to inhibit the actions of GRα, and these actions may increase lipogenesis.
GR Isoforms

Clearly, there is an important role for GRβ in human physiology and disease. However, rigorous testing of the GRβ contribution to GC resistance has not been possible because rodents, especially mice, were thought to not express the β isoform [46]. We have discovered that mice do express GRβ (See Chapter 2). Its discovery has gone unnoticed because investigators before us assumed that the splicing mechanism that generates β in humans must be the same in mice. This is not the case – the alternative splicing event differs in mice. Yet, it still produces a form of β that is similar in structure and functionality to the human form. Mouse GRβ has a C-terminus region that is the same size (15 amino acids) as human β, does not bind dexamethasone (Dex) agonist, is inhibitory of mouse GRα at reporter and endogenous genes, is expressed at the same ratio to GRβ as in human tissues, and is upregulated by inflammatory signals. All of these properties are the same as reported for human GRβ. Additionally, we have very novel data that mGRβ may be involved in metabolic processes. When murine tissue culture cells are treated with insulin, mGRα mRNA expression was unchanged, as expected, but GRβ goes up. Similarly, in mice subjected to fasting-refeeding, a large increase of GRβ was seen in the liver, while mGRα was unchanged. Because GC hormones are potent regulators of glucose and lipid metabolism, and because GCs are broad and chronic antagonists to the actions of insulin, and that regulation of GRβ expression may be an important factor in maintaining tissue sensitivity to insulin, we speculated that adipocyte
hypertrophy, obesity and the metabolic syndrome are possibly states of GC resistance by increasing expression of GRβ.

**FKBP51 and FKBP52 are Modulators of Steroid Receptor Activity.**

GC resistance due to GRβ has been investigated in the immune system of humans. One study, however, showed the existence of GRβ in zebrafish, and this was demonstrated to be inhibitory to the GRα isoform [47]. Studying the prevalence of GC resistance in a mammalian model was difficult, until uncovering that mice generate the GRβ isoform. GC resistance was shown in mammals even though GRβ was not accountable. TPR proteins have been shown to effect steroid receptor activity (Figure 5), and FKBP51 and FKBP52 have been the most extensively studied. Overexpression of FKBP51 was demonstrated to regulate the actions of several steroid receptors; GR, progesterone receptor (PR), MR were all shown to be inhibited [48], however, the androgen receptor (AR) was shown to be positively affected by this co-chaperone [49]. Squirrel monkeys have high circulating cortisol and were shown to have GC resistance by high expression of FKBP51 that significantly reduced the binding affinity of GR to ligand [50]. Later, three New World primates, squirrel, cotton-top tamarin and owl monkies, were all shown to have high expression of FKBP51 and low expression of FKBP52 that resulted in GC resistance [51]. FK506 is an immunosuppressive drug that can bind to both FKBP51 and FKBP52, and
squirrel monkeys treated with this drug were found to derepress the inhibition of FKBP51 and increase GR activity [52].

FKBP52, on the other hand, was shown to positively affect GR activity. Overexpression of FKBP52 in Saccharomyces cerevisiae showed an increase of GR activity and no change with Estrogen Receptor (ER) [53]. FKBP52 KO MEF cells further agreed with this study, in that the loss of this protein resulted in significantly less activity of GR [54]. As with GR, Galigniana et al. demonstrated that FKBP52 also comes into the complex of MR when agonist is bound to interact with the motor protein dynein and translocation to the nucleus occurs [55]. FKBP52 also was shown to regulate AR positively, and the loss of this protein in male mice resulted in hypospadias, reduced penis development, sperm motility and prostate dysgenesis [56] [57] [58]. Female FKBP52 KO mice have similar phenotypes of reduced fertility in that the uterine cannot be stimulated and this reduces reproduction significantly [58]. This was further shown to be due to the positive actions of FKBP52 on PR. To date, what is known about FKBP52 and FKBP51 is that they have inverse modulatory effects on steroid receptor activity, other than AR.

There is very little known about the role TPR proteins have in regulating obesity and their control of steroid hormone receptors on a high fat diet. We have generated FKBP52-deficient mice [58] [59], which are viable at birth and apparently normal into adulthood (except for infertility). Yet, cells derived from FKBP52 KO mice have reduced GR activity. Thus, FKBP52 is not a global
regulator of GR, as such an effect, like GR KO mice, should result in peri-natal lethality [14]. Strikingly, our FKBP52 heterozygous mice, when fed a high fat diet, acquire symptoms similar to the metabolic syndrome; in which they develop hyperglycemia, hyperlipidemia, hyperinsulinemia and weight gain [60]. On the other hand, we have found that FKBP51-deficient mice, when fed high-fat diets, have significantly lower triglyceride plasma levels and lack the ability to store fatty acids in the visceral adipose tissue, possibly due to increased GR activity resulting from loss of inhibitory FKBP51 (data unpublished). The results of the FKBP52 heterozygous mice on a high fat diet suggested that obesity is a state of GC resistance and when the actions of GCs are reduced or inhibited that it results in obesity and the metabolic syndrome. Sensitivity to GCs is critical and protects from diet induced obesity as was seen with FKBP51 KO mice on a high fat diet. The loss of PP5 also results in increased GR transcriptional activity, as well as augmented phosphorylation of GR [61] (see Chapter 3). The effect that FKBP51 and FKBP52 may have on phosphorylation of GR is unknown. Overall, TPR proteins may serve as novel therapeutic targets for obesity and the metabolic syndrome.

**Structure and function of FKBP51, FKBP52, Cyp40, and PP5**

Figure 6 illustrates the functional domain structures of FKBP52, FKBP51, Cyp40 and PP5. Binding by these proteins to the TBD of HSP90 is via the three consecutive TPR domains located in the N-terminal region of PP5 and C
terminus of the other TPR proteins. TPR domains are imperfect 34 amino acid sequences that mediate protein-protein interactions [62]. PP5 is unique among phosphatases because it contains TPR domains and a peptidyl-prolyl isomerase (PPIase)-like domain. Both FKBP52 and FKBP51 have similar PPIase-like domain structures. However, an additional PPIase domain has been observed within their structure that has been shown to bind to the immunosuppressive drug FK506; this domain is also within the structure of Cyp40 and is bound by CsA. Additionally, FKBP52 and Cyp40 contain one other domain, a putative binding site for calmodulin (CaM) that has not been revealed in FKBP51 or PP5. The ability of FKBP51/FKBP52 and Cyp40 to bind the immunosuppressive drugs FK506 and cyclosporine A (CsA) has served to categorize these proteins as immunophilins. However, it is because all of these proteins, including PP5, contain TPR domains that we refer to them as TPR proteins.

Figure 6. Structural domains of TPR proteins. See text for details.
Although the contribution of FKBP52 and FKBP51 to GR cellular function has been much investigated, very little is known of PP5 actions on GR or any other steroid receptor. It is widely speculated that PP5 interacts with most members of the steroid receptor family; however, direct evidence only exists for the ER [63], MR [64] and GR [65]. In all cases, interaction with PP5 is mediated by HSP90 [15]. PP5 may regulate the intrinsic ability of GR to bind glucocorticoids, as GR complexes containing PP5 have higher binding affinity for glucocorticoids compared to complexes containing FKBP51 [17]. Furthermore, PP5 may control nuclear translocation of GR due to its ability to interact with the motor protein dynein via its PPIase-like domain [66].

**PPARγ can bind HSP90**

TPR proteins have been shown to bind to nuclear receptors of the steroid family, however this does not hold true for all nuclear receptors. The interaction of TPR proteins with steroid receptors is mediated by the chaperone HSP90. Some nuclear receptors have been demonstrated to not bind to the HSP90 chaperone complex, such as thyroid and retinoic acid receptors [67]. Similarly, orphan receptors were thought to not bind the HSP90 chaperone machinery, such as LXR, PPARα and PPARγ that are involved in lipid metabolism, which would make the binding cleft for TPR proteins. Surprisingly, Sumanasekera et al. showed that all three isoforms of the PPARs (α, β and γ) bind to HSP90 [68]. The study also showed that a dominant negative overexpression of the TPR domain
of PP5 could regulate activity of the PPARs. Phosphorylation of PPARγ has been shown to be inhibitory; on the other hand, phosphorylation was demonstrated to positively affect GR activity. Therefore we postulated that PP5 may work as a fulcrum to regulate lipolysis and lipogenesis via removing phosphates from PPARγ and GR, by binding to the HSP90 chaperone complexes (See Chapter 3).

**Structure and function of PP5 and lipid activation via TPR domains**

A diagram showing pertinent features of PP5 and its isoforms are seen in Figure 7. The phosphatase domain resides in the C-terminal region and contains all the relevant motifs of the PPP family of phosphatases [69] [70] [71]. PP5, unlike other phosphatases, displays low phosphatase activity under normal conditions [70] that may result from an inhibitory interaction between the C-
terminus (residues 490-499) and the TPR domain [72]. Residues E76 in the second TPR motif and Q495 in the C-terminus are critical to the inhibitory interaction [72] [71]. Such intrachain interactions may allow the TPR domain to mediate phosphatase activity by binding and shielding it from activation. The phosphatase domain can be activated when the TPR domains are bound or truncated from PP5 [72] [71]. It is for this reason that stimulation of PP5 can occur with fatty acids, such as arachidonic acid [73], which bind the TPR domain of PP5 to initiate phosphatase activity [74]. Saturated fatty acids and fatty acid esters did not stimulate phosphatase activity in this study [70] [74]. TPR proteins may aid in FA metabolism, export and/or storage. A study in Drosophila melanogaster demonstrated that the adp gene knockout resulted in significant increase of FA storage [75]. The adp gene encodes a protein that contains three consecutive TPR domains that can bind long chain fatty acids, similar to PP5 [75]. The loss of this gene resulted in adipocyte hypertrophy and obesity [75]. This suggest a possible role of TPR proteins in aiding in lipid storage or metabolism.

Later we show in Chapter 3 that PP5 is essential for fatty acid uptake, PPARγ activity and its expression in adipose is regulated by a high fat diet. The involvement of TPR proteins during adipogenesis and lipogenesis and their actions on GR after differentiation into adipocyte has not been documented. However, one study demonstrated that FKBP51 is upregulated during adipogenesis, and GR and FKBP52 downregulated [76] [77] [78]. We therefore speculate that PP5 and FKBP51 may be involved in storage of fatty acids and
lipogenesis, and FKBP52 in lipolysis via their actions on GR, most likely via TPR domains. TPR proteins may be working to regulate lipolysis through GR and lipogenesis via PPARγ, and their actions are mediated through HSP90. Therefore, glucocorticoid resistance can occur by several mechanisms; increased expression of GRβ, PP5, FKBP51 and decrease of FKBP52 that are inhibitory to the lipolytic actions of GRα (Figure 5).
Chapter 2

Discovery of Glucocorticoid Receptor-β in Mice with a Role in Metabolism

(Published in Molecular Endocrinology 2010 Sep;24(9):1715-27)

Terry D. Hinds, Jr., Sadeesh Ramakrishnan, Harrison A. Cash, Lance A. Stechschulte, Garrett Heinrich,
Sonia M. Najjar and Edwin R. Sanchez*

Center for Diabetes & Endocrine Research (CeDER)
and the Department of Physiology & Pharmacology,
University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614, USA.

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*Corresponding author: Department of Physiology & Pharmacology, University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614-5804, (419) 383-4182, FAX (419) 383-2871, edwin.sanchez@utoledo.edu.

Abbreviated title: Murine Glucocorticoid Receptor Beta

Keywords: glucocorticoid receptor beta, mouse, metabolism, inflammation, steroids, nuclear receptor

This work was supported in part by National Institutes of Health grants DK70127 (to E.R.S.) and DK54254 (to S.M.N.), and by a United States Department of Agriculture grant 38903-02315 (to S.M.N.). Terry Hinds, Jr. was supported by a predoctoral NIH National Research Service Award (F31DK84958).

Disclosure Statement: The authors have nothing to disclose.
Glucocorticoid hormones (GCs) control diverse physiological processes, including metabolism and immunity, by activating the major glucocorticoid receptor (GR) isoform, GRα. However, humans express an alternative isoform, hGRβ, that acts as an inhibitor of hGRα to produce a state of glucocorticoid resistance. Indeed, evidence exists that hGRβ contributes to many diseases and resistance to GC therapy. Yet, rigorous testing of the GRβ contribution has not been possible because rodents, especially mice, are not thought to express the β isoform. Here, we report expression of GRβ mRNA and protein in the mouse. The mGRβ isoform arises from a distinct alternative splicing mechanism utilizing intron 8, rather than exon 9 as in humans. The splicing event produces a form of β that is similar in structure and functionality to hGRβ. Mouse GRβ has a degenerate C-terminus region that is the same size as hGRβ. Using a variety of newly-developed tools, such as a mGRβ-specific antibody and constructs for over-expression and shRNA knockdown, we demonstrate that mGRβ cannot bind dexamethasone agonist, is inhibitory of mouse GRα, and is upregulated by inflammatory signals. These properties are the same as reported for hGRβ. Additionally, novel data is presented that mGRβ is involved in metabolism. When murine tissue culture cells are treated with insulin, no effect on mGRα expression was observed, but GRβ was elevated. In mice subjected to fasting-refeeding, a large increase of GRβ was seen in the liver, while mGRα was unchanged. This work uncovers the much-needed rodent model of GRβ for investigations of physiology and disease.
Human glucocorticoid receptor (hGR) is expressed as two major isoforms: hGRα and hGRβ (1, 2). Glucocorticoid hormones (GCs) control diverse physiological processes (3, 4), such as metabolism, immunity/inflammation, development and behavior. These responses are a direct result of GRα activity as a hormone-activated transcription factor (5, 6). In contrast, the role of GRβ in GC control of physiology is still poorly understood. Most recent studies suggest that GRβ acts as an inhibitor of GRα (7-10) to produce a state of glucocorticoid resistance (1, 2). Indeed, there is indirect evidence that elevated expression of GRβ may be responsible for a variety of immunological diseases. Severe asthma, leukemia, ulcerative colitis, chronic sinusitis, systemic lupus erythematosus, and possibly cigarette smoking, all correlate with over-expression of GRβ (2, 11-13). Many patients suffering from these diseases are refractory to GC treatment. Not surprisingly, increased activation of pro-inflammatory transcription factors and cytokines has also been noted in cases of GC resistance with elevated GRβ expression. These observations suggest an important role for GRβ as a homeostatic mechanism in the normal attenuation of GC responses and as a possible culprit in hormone-resistant disease states.

The human GR gene was cloned and sequenced in 1985 revealing the expression of hGRα and hGRβ (14). Additional studies showed that the isoforms result from alternative splicing to yield GRs identical through amino acid 727 but which differ in their carboxyl-terminal regions. The hGRα carboxyl-terminus is composed of 50 amino acids containing important sites for hormone binding, as
well as helix 12, which provides critical transcriptional activation activity as a site for co-regulator interaction (15). In contrast, the unique and non-homologous carboxyl-terminus of hGRβ is a disordered 15 amino acid region of no known function. Not surprisingly, hGRβ cannot bind GC agonists (7, 16), however, binding by RU486 antagonist, although disputed (17), has been shown by one laboratory (18). Although hGRβ contains AF-1 and DNA-binding domains identical to those in hGRα, no transcriptional activation or repression activities in response to hormone have yet been found for this isoform. Instead, most data point to hGRβ as an inhibitor of hGRα activity, either through competition for co-regulators or through formation of inactive α/β heterodimers. Consistent with this mechanism is the predominant presence of hGRβ in the nucleus of most cells, whereas hGRα resides in the cytoplasm, undergoing nuclear translocation in response to ligand (19). Thus, hGRβ can be viewed as a dominant-negative inhibitor of hGRα, a mechanism of action which may underlie the potential role of GRβ in GC resistance. However, two recent studies using gene array analyses have revealed that hGRβ can constitutively regulate genes not controlled by hGRα (17, 18). Therefore, hormone-free hGRβ, in addition to its dominant-negative activity, appears to have an intrinsic gene regulatory function important to physiological responses distinct from hGRα.

The only observation of GRβ outside humans has been in zebrafish (20). Yet, when the mouse GR was originally cloned and sequenced, one active GR was discovered that responded to GCs (21), but two different mRNAs were
found with distinct poly-A tails (22). Moreover, an intact mGR protein was identified which was unable to bind hormone (23). Curiously, the alternative isoform of mouse GR was not pursued, and it is now generally accepted that rodents do not express GRβ. This conventional wisdom owes its existence to studies designed to discover mGRβ based on the hGRβ process. In humans, GRα and GRβ share exons 1–8 but diverge to contain exons 9α and 9β, respectively, based on alternative usage of splice acceptor sites in exon 9 (24). Efforts to discover GRβ based on similar splicing events in rodents and sheep have been unsuccessful (25, 26). The recent discovery of GRβ in zebrafish has shown that splicing can also occur, not in exon 9, but through alternative donor sites in intron 8, to yield a zGRβ with properties similar to human GRβ. In this work, we demonstrate the existence of GRβ mRNA and protein in the mouse. Like zebrafish GRβ, mouse GRβ results from alternative usage of donor sites, in this case in a region previously assigned as intron 8 of the mGR gene. Although the splicing mechanism differs from that which generates hGRβ, mGRβ contains a 15 amino-acid carboxyl-terminus that is identical in length and highly similar in sequence to hGRβ. In contrast, the C-terminus of zGRβ is much larger (50 amino acids). Mouse GRβ shares similar properties with hGRβ, including ubiquitous expression in mouse organs, lack of responsiveness to the GC agonist dexamethasone, and an ability to inhibit mGRα activity. This study has uncovered the much-needed rodent model of GRβ, providing a new tool for
future *in vivo* investigations of glucocorticoid resistance and sensitivity in physiology and disease.

**Results**

**The Mouse GRα and GRβ Isoforms**

To identify the mGRβ gene, the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) was used for the Nr3c1 nuclear receptor subfamily 3, group C, member 1 (*Mus musculus*), and information was downloaded for the complete GR genomic DNA sequence. Information regarding introns and exons in the mouse GR gene (ENSMUSG00000024431) was downloaded from Ensembl Mouse GeneView (www.ensembl.org). A single mouse GR genomic sequence was identified. The mGR gene is located on chromosome 18 and consists of at least nine exons (Fig. 1). The Drosophila website for splice site predictions (http://www.fruitfly.org/seq_tools/splice.html) was used to characterize possible acceptor and donor alternative splice sites within mGR (see supplementary Figs. 1S and 2S). *In silico* mapping revealed that intron 8 was the most likely target of alternative splicing, yielding several donor and acceptor sites with high splice-site prediction scores. Interestingly, no splice-site predictions were found in exon 8. Predicted splice sites were also found in exon 9 – in the distal region of the exon where alternative splicing is known to occur in the hGR gene (7, 16). For initial screening of mGRβ, primers were designed to
test for utilization of the intron 8 and exon 9 sites, followed by reverse transcriptase-PCR (RT-PCR) analysis of total RNA from mouse embryonic fibroblast (MEF) cells (Fig. 1A). Consistent with prior published efforts (25), no PCR product was observed using a primer specific to the distal region of exon 9 (Fig. 1B). However, all three primers in the intron 8 region yielded PCR products, suggesting the existence of mGR transcripts derived from intron 8.

To determine the presence of full-length transcripts, RT-PCR was performed on MEF cell total RNA using a common forward primer at the 5′ end of exon 1 and reverse primers to sequences within intron 8 (primer #1) or to the proximal region of exon 9 (primer #2) (Fig. 2A). As expected, a full-length transcript was seen corresponding to mGRα (Fig. 2B). More importantly, a full-length transcript containing sequences derived from intron 8 was also found. The intron 8-containing mRNA had reduced abundance relative to mGRα, consistent with the known relationship of hGRβ and hGRα (16). To sequence the novel full-length product, RT-PCR was repeated using a forward primer from the ATG start site in exon 2 and a reverse primer (#3) specific to a more distal portion of intron 8 and the product was cloned and sequenced (GenBank accession # HM236293). The results (Fig. 2C) showed presence of coding sequences that extend beyond exon 8 into intron 8, with a stop codon at position 46-48 base pairs within the intron (for complete mRNA sequence see supplementary Fig. 3S). Additional non-coding sequences derived from the distal portion of intron 8 were present. These results suggest that the β-specific
amino acids of mGR are located in the proximal portion of intron 8, in contrast to humans, where GRβ sequences are found in the distal portion of exon 9. It is likely, therefore, that mGRβ arises through use of alternative donor sites located within intron 8, as opposed to the hGR mechanism of alternative acceptor sites in exon 9.

The translation product of mGRβ spans 748 amino acids (Fig. 3A). Alignment of mGRβ with the mGRα peptide sequence revealed 93% homology. The encoded mGRβ protein diverges from mGRα at amino acid 733, beyond which is a disordered C-terminus of 15 amino acids. In comparison, hGRα and hGRβ are also 93% identical. Interestingly, both hGRβ and mGRβ add an additional 15 amino acids to exon 8, even though the means of alternative splicing are different. The two β isoforms are an overall 87% match and share a functional domain organization that appears identical (Fig. 3B). In both hGR and mGR, the point of divergence between α and β isoforms is located in the transition region between the 10th and 11th helix of the ligand-binding domain (7, 16). This results in a degenerate helix 12 in the ligand-binding domains of hGRβ and mGRβ. As a consequence, mGRβ, like hGRβ, is unresponsive to the glucocorticoid agonist dexamethasone (see below).

**Expression of mGRβ mRNA In Vivo**

As a first test of significance, expression of mGRβ in mouse organs and tissues was assayed by quantitative real-time PCR (qPCR). Total RNA was
extracted from mouse tissues and analyzed with forward primers to exon 8 and reverse primers for exon 9 (mGRα) and intron 8 (mGRβ) indicated in Fig. 2. The qPCR results are from two different C57/Bl6 mice. The data are expressed relative to mGRα expression. In all tissues studied, mGRα and mGRβ mRNA appear to be co-expressed, with mGRα mRNA levels being significantly higher than mGRβ (Fig. 4). The highest level of mGRβ was observed in the spleen, with lesser amounts in kidney and liver. These results are in good agreement with expression profiling of GRβ mRNA in human (16) and zebrafish (20) tissues. Moreover, the high levels of GRβ in mouse spleen are consistent with the important role played by human GRβ as an dominant-negative inhibitor of GRα in lymphoid cells (10).

Characterization of mGRβ Protein

A cDNA expression vector was made by cloning the full-length mGRβ PCR product (Fig. 2) into pcDNA3.1 to generate the pMGRβ-H57 vector (Fig. 5A). Receptor-less COS-7 cells were transfected with equal amounts of pMGRβ-H57 and pSV2Wrec encoding mGRα, followed by Western-blotting using FiGR monoclonal antibody against mGR that recognizes a shared epitope in the N-terminal domain (27)(see Fig. 3). The results (Fig. 5A) show expression of mGRβ protein with a molecular mass smaller (81.7 kDa) than mGRα (86.0 kDa). Importantly, this shows that the endogenously produced mGRβ mRNA can be translated into protein. To detect endogenous mGRβ, a rabbit polyclonal
antibody (rMGRB-Ab) was made. Specificity of rMGRB-Ab was determined by transfecting COS-7 cells with pSV2Wrec, pMGRβ-H57 or empty vector, followed by probing of Western blots with rMGRB-Ab and FiGR antibodies. Results were analyzed with the Odyssey infrared system utilizing red- and green-emitting counter antibodies to detect FiGR and rMGRB-Ab, respectively. As predicted, rMGRB-Ab detected mGRβ, but showed no reactivity to mGRα (Fig. 5B). To demonstrate existence of endogenous mGRβ protein in cells, untransfected MEF cell lysates were immunoadsorbed with rMGRB-Ab or FiGR followed by Western-blotting (Figs. 5C,D). The results show immune-specific pull-down of mGRβ by the rMGRB-Ab and FiGR antibodies. As expected, the FiGR purification results showed expression of mGRβ protein to be lower than mGRα. As a last test, indirect immunofluorescence with rMGRB-Ab was performed in MEF cells (Fig. 5E), showing localization of mGRβ protein in the cytoplasm and nuclear foci. Interestingly, Dex treatment elevated the mGRβ signal in both compartments. This result is consistent with the ability of Dex to increase mGRβ mRNA expression (see Fig. 8 below).

**mGRβ is a Hormone-insensitive Dominant-negative Inhibitor of mGRα**

Because mGRβ and hGRβ express C-terminal domains that are almost identical, we reasoned that mGRβ would be unresponsive to activation by glucocorticoid ligands. In addition, Cidlowski and colleagues have shown that the ability of hGRβ to inhibit hGRα activity on gene expression is encoded by its
C-terminal 15 amino acid domain (9). Thus, the mGRβ discovered in this work seemed likely to act as a hormone-insensitive, dominant-negative inhibitor of mGRα. To test both of these functions, COS-7 cells were transfected with pGRE2E1B-Luc reporter and various combinations of pMGRβ-H57 and pSV2Wrec. The results of Fig. 6A showed that mGRβ cannot increase pGRE2E1B-Luc activity in response to Dex or RU486. RU486 antagonist was chosen because of one report suggesting reactivity to this ligand by human GRβ (18). Fig. 6B demonstrates dose-dependent inhibition of mGRα activity by mGRβ that begins at a β:α molar ratio of 0.5:1. To determine the dominant-negative activity of mGRβ at endogenous genes, real-time PCR analysis was performed in mGRα-expressing MEF cells following transfection of pMGRβ-H57 (Fig. 6C). The results showed that Dex-induced expression of glucocorticoid-inducible leucine zipper (GILZ), serum- and glucocorticoid-inducible kinase (SGK), pyruvate dehydrogenase kinase-4 (PDK4), and glucose-6-phosphatase (G6Pase) were effectively inhibited by mGRβ. In contrast, mGRβ had no effect on expression of FKBP51, and on the ability of Dex to down-regulate mGRα expression. The latter results suggest that mGRβ is not a global inhibitor of mGRα actions, and that the inhibitory effect of mGRβ is not due to decreased mGRα expression.

To directly test the inhibitory actions of endogenous mGRβ, short-hairpin RNA specific to mGRβ was designed based on its unique intron 8 sequence. MEF cells were infected with lentiviral constructs expressing mGRβ shRNA or empty vector. Fig. 7A demonstrates that the mGRβ shRNA effectively blocks expression
of the mGRβ full-length mRNA, while leaving mGRα mRNA untouched. More importantly, Dex-induced expression of three endogenous genes (PDK4, G6Pase and GILZ) was increased in response to mGRβ knockdown (Fig. 7B). These results are the first direct evidence that endogenously-expressed GRβ (mouse or human) plays a functional role in mGRα actions. Consistent with the results of Fig. 6C, mGRβ knockdown had no effect on FKBP51 expression.

**Hormonal and Dietary Control of mGRβ Expression**

As an agonist to mGRα, dexamethasone not only causes activation or repression of GR-regulated genes, it also causes autologous down-regulation of GRα expression in many species as a means by which to attenuate over-stimulation by GC ligands (28, 29). Because GRβ can be viewed as an alternative mechanism of GRα attenuation, and as GRβ cannot bind Dex, we determined what effect Dex had on mGRβ expression. MEF cells were treated with 100nM Dex for two h, followed by measurement mGRα and mGRβ mRNA expression via qPCR (Fig. 8A). As expected, a significant decrease in mGRα mRNA was seen in response to hormone treatment. Interestingly, mGRβ mRNA was increased. This suggests the existence of a negative feedback loop, most likely mediated by GRα, that up-regulates GRβ expression to control sensitivity of cells to glucocorticoids.

Glucocorticoid hormones acting through hGRα are potent anti-inflammatory drugs that inhibit the ability of NF-κB to activate expression of
pro-inflammatory cytokine genes (30). In turn, activation of NF-κB by tumor necrosis factor (TNF)-α leads to reciprocal attenuation of hGRα transcriptional activity (31). Although NF-κB can inhibit hGRα through direct protein-protein binding (30), an additional mechanism was recently discovered in which NF-κB causes selective up-regulation of hGRβ expression (10). To test whether this mechanism applies to mouse mGRβ, we measured mGRα and mGRβ expression in RAW 264.7 monocytic macrophage cells subjected to TNF-α treatment (Fig. 8B). The results show a modest ~1.5-fold induction of mGRα at the 8 h time-point, but a greater ~2.5-fold induction of mGRβ. These data are in good agreement with those of Webster et al (10), and suggest that mGRβ, like its human cognate, serves to induce a state of GC resistance to maximize inflammatory responses.

As a last test of relevance, we asked if mGRβ might play a role in GC control of metabolism. Glucocorticoids are well-known antagonists to insulin that promote gluconeogenesis over glycogenesis, especially in the liver (4, 32). Conversely, insulin acts to inhibit gluconeogenesis, in part, by blocking GR activity at the hepatic phosphoenolpyruvate carboxykinase (PEPCK) promoter (33, 34). Because mGRβ can similarly block mGRα activity at the PDK4 and G6Pase genes (Fig. 6C), we tested whether insulin might achieve this effect on gluconeogenic genes by up-regulating expression of mGRβ (Fig. 8C). The results show a short-lived increase of mGRα immediately after insulin treatment of MEF cells, but a more dramatic increase of mGRβ with longer treatment. As a further
test, we assayed for mGR isoform expression in the livers of mice subjected to a fasting-refeeding regimen (Fig. 8D). During refeeding, there is a well-documented and robust stimulation of hepatic metabolism by insulin (35, 36). The results show a small increase in mGRα during the early stages of refeeding, but a much larger increase in mGRβ at the later stages. Taken as a whole, these results represent a potential new role for GRβ (mouse or human) in the GC-insulin axis in which upregulation of GRβ may serve to maintain insulin sensitivity.

**Discussion**

Due to underlying pathology or drug treatment, glucocorticoid resistance can develop and is now a major concern in many disease states. Thus, the need for a mammalian GRβ model is imperative. In humans, GC resistance can occur by two major mechanisms: loss-of-function mutations in GRα (37), or by increased expression of GRβ, which acts as a dominant-negative inhibitor of GRα. Although GRα mutations can result in a type of GC resistance that is both systemic and severe, these mutations are rare. In contrast, the evolving evidence suggests that GC resistance based on GRβ is much more common and likely to be tissue-specific in nature. To date, GC resistance based on GRβ has been principally characterized in immunological diseases and drug-resistant states. Immune system homeostasis is balanced by glucocorticoids, which regulate immune cell turnover by suppressing cytokine production and
promoting apoptosis. GC insensitivity due to elevated human GRβ expression increases proinflammatory cytokines, leading to escalated cell growth and reduced cell death (38). Super-antigens, such as staphylococcal enterotoxin B and toxic shock syndrome toxin, have been demonstrated to cause increased GRβ expression and GC resistance (39). As well, proinflammatory cytokines, such as TNFα and IL-1, increase expression of GRβ via the NF-kB pathway (10).

Although cytokine production is increased in all asthma patients, some subjects do not benefit from GC therapy because of elevated GRβ (40, 41). Indeed, fatal asthma has been linked to extremely high levels of GRβ in the airways (42) and a complete loss of GC drug response (43). Other inflammatory diseases linked to high levels of GRβ include: ulcerative colitis, ankylosing spondylitis (44); cigarette smoking (45); leukemia (12); and systemic lupus erythematosus (46).

GC resistance via GRβ is almost unknown in diseases of metabolism. Only one study on this topic has been published. It showed that exposure of skeletal muscle to GCs leads to a decline in GRα expression and a concomitant increase of GRβ (47). Because GC hormones are potent regulators of glucose and lipid metabolism, and because GCs are broad and chronic antagonists to the actions of insulin (48), we reasoned that GRβ may play a role as a modulator of GRα actions, similar to insulin antagonism. In this work, we provide evidence that mGRβ is indeed involved in metabolic processes. GC induction of the gluconeogenic enzymes PDK4 and G6Pase was inhibited by mGRβ, suggesting that one function of the GRβ isoform, like insulin, is to block glucose production.
Not surprisingly, in cells treated with insulin, mGRα expression was unchanged, but mGRβ went up. In cells treated with Dex, mGRβ was also increased, suggesting that both GCs and insulin share upregulation of mGRβ as a common mechanism for antagonism of mGRα. Similarly, in mice subjected to fasting-refeeding, a large increase of GRβ was seen in the liver, while mGRα was once again unchanged. The fasting-refeeding regimen employed is known to produce a robust stimulation of hepatic metabolism by insulin (35, 36). Taken as a whole, these data are the first evidence that GRβ upregulation may be an important mechanism for maintaining organ sensitivity to insulin.

In this work, we present data that mice express a GRβ isoform that derives from alternative splicing of intron 8, similar to the mechanism in zebrafish (20). The sequence encoding the GRβ-specific amino acids is located in the middle portion of exon 9 in the human gene, but is found in intron 8 in the zebrafish gene. In zGR, exon 9 is skipped or silenced as a result of alternative splicing, and intron 8 is retained. Therefore, hGRα and hGRβ mRNA are produced through alternative usage of a splice acceptor site in exon 9, whereas alternative use of a splice donor site in intron 8 appears to be the underlying mechanism in both mice and zebrafish. This mechanism is often referred to as intron retention and is not unique to mGRβ. Indeed, C-terminal isoform variants of vitamin D, peroxisome proliferator-activated (PPAR)-α and PPAR-γ receptors can be generated by intron retention (24). It has long been thought that GRβ does not exist in rodents, in large part because one high-profile study concluded that the
alternative splicing event does not occur in mice (25). The study assumed that the splicing event in mice must be similar to humans and used primers that focused on the distal portion of exon 9. It is now clear that mice express a GRβ isoform derived from intron 8. In contrast to zGRβ, which has a 50 amino acid C-terminal region, mGRβ has a protein structure in which the C-terminal region is the same size (15 amino acids) as the human β isoform. Moreover, for the properties so far tested, mGRβ is highly similar to hGRβ.

The mechanism controlling alternative splicing of the GR gene is poorly understood but is generally thought to involve the generation of a spliceosome composed of ribonucleoproteins and serine-arginine (SR) rich proteins, among others, that bind structures found in both introns and exons, such as branch point sequences (BPS) and polypyrimidine tracts (PPT). [For review, see ref. (49)]. Intron 8 in mice is nearly twice as large as intron 8 in humans (1061 bp vs 526 bp, respectively). As intron size correlates to the likelihood of alternative splicing, this reason alone may account for why murine species utilize intron 8 for isoform control. Interestingly, we have identified two BPS sites within murine intron 8, as well as a single PPT (supplementary Fig. 1S). We speculate that mGRα and β are generated through a mechanism that uses the separate, but distinct, BPS sites to initiate splicing. Targeting of these sites or the spliceosome proteins that bind them may eventually form the basis by which to inhibit mGRβ expression, resulting in cells and tissues that are more sensitive to glucocorticoids. Recent advances lend credence to this hypothesis. Bombesin, a
ligand for G protein-coupled receptors, is known to upregulate expression of SRp30c, causing elevated expression of GRβ in prostate cancer cells (50). A related protein, SRp40, regulates splicing of GRβ in HeLa and 293T cells (51). As can be seen by these examples, major factors that regulate spliceosome action on the GR gene are only now being discovered. Our newly-discovered mouse model of GRβ can now be used to establish feasibility of these targets for eventual alteration of human GRβ expression.

Our data show that mGRβ expressed in receptor-less COS cells cannot respond to Dex or RU486 by activating expression of a pGRE-Luc reporter. This result is in good agreement with most studies of hGRβ showing that it cannot bind GC agonists, nor activate reporter or endogenous gene expression in response to hormone (7-9, 16). However, there is one report demonstrating hGRβ binding by RU486 with consequent induction of nuclear translocation (18). In contrast to this unresolved issue, it is now clear that hGRβ can exert constitutive control on gene expression (17, 18). These studies utilized gene array approaches following over-expression of hGRβ and showed that, in addition to its ability to suppress hGRα-regulated genes, hGRβ exerted both positive and negative control over a unique set of genes not regulated by hGRα. In addition, a constitutive ability of hGRβ to induce histone deacetylation has been found (52, 53), providing a possible mechanism for hGRβ-mediated repression of gene expression. Taken as a whole, these results show that hormone-free GRβ has an unexpected constitutive and intrinsic gene expression function that may regulate
cellular and physiological responses distinct from GRα. Although it remains to be seen whether mGRβ can replicate the latter property, the mGRβ mouse model described here is likely to foster study of glucocorticoid resistance and sensitivity in diverse disease states, such as inflammation, hematological cancers, diabetes and obesity.

Materials and Methods

In Silico Prediction of Mouse GRβ. To identify mouse GRβ the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) was used for Nr3c1 nuclear receptor subfamily 3, group C, member 1 (Mus musculus), and gene information was downloaded for the complete GR genomic DNA sequence. Information regarding introns and exons (ENSMUSG00000024431) was downloaded from Ensembl Mouse GeneView (www.ensembl.org). A single mouse GR genomic sequence was identified. The NCBI website was used to search for expressed sequence tag and cDNA sequences derived from mouse GR transcripts. Text searches and BLAST searches were performed. BLAST searches of the mouse genomic sequence were carried out using the BLASTN tool at the Sanger Institute Ensembl server.

Characterization of Alternative Splice Sites Within Mouse GR Gene. Recognition of alternative splice sites in exons 7-9 and intron 8 of the mouse GR gene were investigated using the Drosophila website for splice site predictions.
Sequences with the highest scores in exon 8, exon 9 and intron 8 were identified as potential targets for alternative splice sites that could lead to production of mGRβ (Fig. 1S). Primers were developed for these prospective splice sites (see below), and an initial screen for mGRβ was performed via RT-PCR.

**Initial Screening of mGRβ.** Total RNA was isolated from MEF cells using 5 Prime PerfectPure RNA Cell Kit (Fisher Scientific Company, LLC) according to the manufacturer’s instructions. Total RNA concentration and purity was determined by measuring absorbance at 260/280 nm and confirmed on an RNA denaturing formaldehyde gel. Purified RNA (1 μg) was used to produce complementary strands of DNA (cDNA) using a 1st strand synthesis kit (Roche Applied Science, Indianapolis, IN). Newly synthesized DNA (3 μl) was amplified by RT-PCR using forward primers containing sequences from exon 7 (GCAGAGAATGACTCTACCCTGCA) and reverse primers based on prospective splice sites ratings from the Drosophila website. Three different reverse primers for intron 8 (TAAAGGCATCTGACCACCACC, CTGTCTTTGGCCTTTTGAGATAGG, and CTTTGGCCTTTTGAGATAGGATC) and two different reverse primers for the latter part of exon 9 (TCCCCAGCTCCCTCTCCCTAG and TCCCTCTCCCTAGCTTAGAG) were used to identify the location of mGRβ (see Fig. 1). 18S RNA was amplified as an internal control. PCR conditions used were: 95°C for 5 min, 95°C for 1 min, 60°C
for 1 min, 72°C for 40 seconds, and 72°C for 10 min. PCR products were electrophoresed in a 1% agarose gel and visualized with ethidium bromide. The 1 Kb Plus DNA Ladder (Invitrogen) was used as a size standard.

**Generation of Complete mGRβ Transcript.** Primers for exon 1 (GTAGAGACGAAACTCCAGCA) and reverse primers were based on sequences from intron 8 (GRβ) (TAAAGGCATCTGCCACCACC) and exon 9 (GRa) (AGCTAAGGAGATTCTTCAACCACA) of mGR and utilized to demonstrate the complete mGR α and β mRNA constructs. The expected mGR α and β products were 2251 and 2361 bp, respectively. PCR conditions used were: 95°C for 5 min, 95°C for 1 min, 60°C for 1 min, 72°C for 3.5 min, and 72°C for 10 min.

**Cloning and Sequencing of mGRβ.** Cloning and sequencing of mGRβ from MEF cells was performed as follows. Following total RNA isolation, cDNA synthesis was achieved using KOD Xtreme Hot Start DNA Polymerase (Novagen, Madison, WI) and a forward primer for the ATG start site of the open reading frame in exon 2 (CGGGATCCATGGGACTGTATATGGGAGAG) and a reverse primer to the distal portion of intron 8 (GCTCTAGAGTAATGTATCTTGATTGTGGC). The expected product was 2852 bp. GRβ PCR products were ligated to pcDNA 3.1+ vector using BamHI and XbaI and transformed into One Shot INV F cells (Invitrogen). Plasmid DNA from
positive clones was determined by RT-PCR and further extracted using the Qiagen Spin Miniprep Kit (Qiagen, Crawley, United Kingdom). BamHI and XbaI sites flank the position on the vector at which the mGRβ gene is inserted. The presence of GRβ in mice was confirmed by restriction digestion with BamHI and XbaI to determine the size of the insert (Boehringer Mannheim) and further digested with HindIII to determine sequence specificity. Sequencing was performed by the University of Iowa DNA Facility (Iowa City, Iowa) using T7 forward and BGH reverse primers that flank the gene insertion site of the plasmid. The sequence confirmed plasmid was named pMGRβ-H57. The mGRβ sequence has been deposited to GenBank under accession no. HM236293.

**Quantitative Real-Tme PCR Analysis.** Total RNA was extracted from mouse tissues using 5-Prime PerfectPure RNA Tissue Kit (Fisher Scientific Company, LLC). Total RNA from MEF cells was extracted as described above. cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). PCR amplification of the cDNA was performed by quantitative real-time PCR using qPCR Core kit for SYBR Green I (Applied Biosystems). The thermocycling protocol consisted of 10 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 61°C, and 20 sec at 72°C and finished with a melting curve ranging from 60-95°C to allow distinction of specific products. Primers were designed using Primer Express 3.0 software (Applied Biosystems) to amplify a region in intron 8 that was revealed in the initial screening for mGRβ. A common forward primer in exon 8
(AAAGAGCTAGGAAAAAGCCATTGTC) was used in conjunction with a reverse primer in intron 8 for mGRβ (CTGTCTTTGGCCTTTTGAGATAGG), or a reverse primer in exon 9 for mGRα (TCAGCTAACATCTCTGGGAATTCA). Normalization was performed in separate reactions with primers to 18S mRNA (TTCGAACGTCTGCCCTATCAA and ATGGTAGGCACGGCGACTA). To study whether genomic sequences were amplified, a control sample was used in which no reverse transcriptase was added (non-RT control). The following primers were used for endogenously expressed genes: SGK forward GAGAAGGATGGGCTGAACGAT reverse CGGACCCAGGTTGATTTGTTGA. GILZ forward AATGGCCGGACGGATG reverse GGACTTCACGTTGTGGGACA. PDK4 forward TTTCTCGTCTCTACGCCAAG reverse GATACACCAGGTTGATTTGTTGA. G6Pase forward TGCAAGGGAGAATCAGCAA reverse GGACCAAGGAAGCCACAATG. FKBP51 forward GCTGGCAAAACAACACGAGAG reverse GAGGAGGGCCGAGTTCCAT.

**Generation of mGRβ Antibody.** A rabbit polyclonal antibody to mGRβ via the method used to produce antibody to hGRβ [33]. A peptide corresponding to amino acids 733 - 748 (VSTKHKSKTAKKKK) at the C-terminus of the mGRβ protein was synthesized and purified by Pacific Immunology (Ramona, CA). An N-terminal cysteine was added to the peptide as a linker, followed by conjugation to a peptide carrier protein keyhole limpet hemocyanin and
adjuvant-based immunization in a female New Zealand White rabbit. Pre-immune serum was collected before injecting the rabbits with mGRβ conjugate peptide. The rabbits were boosted two weeks after injection with the mGRβ peptide with Complete Freund’s Adjuvant, and subsequently boosted two more times with Incomplete Freund’s Adjuvant every two weeks. Serum was collected at two months and analyzed via ELISA for mGRβ specific antibodies. Serum of high titer was obtained and subjected to one round of affinity purification using the mGRβ peptide.

**Generation of mGRβ shRNA Lentiviral Construct.** To identify an siRNA to knockdown mGRβ a free Web-based tool ([http://www.genelink.com/sirna/shRNAi.asp](http://www.genelink.com/sirna/shRNAi.asp)) was used to design a putative siRNA against the mGRβ and to design oligonucleotides that encode a corresponding small hairpin RNA (shRNA). The resulting shRNA recognized a sequence beginning at exon 8 within the mouse GR mRNA and extended into intron 8. XbaI and XhoI restriction sites were added to flanking regions of the sequence. Oligonucleotides were:

GGACTCCATGCATGATGTAAGTACCAAACATCAAGAGTGTTTGGTACTTA

CATCATGCATGATGTAAGTACCAAACATCAAGAGTGTTTGGTACTTA

CATCATGCATGATGTAAGTACCAAACATCAAGAGTGTTTGGTACTTA

and the homologous sequence. Synthetic oligonucleotides were annealed, digested with restriction enzymes and then ligated into the XbaI/XhoI sites of the FG12 vector that has an independent GFP marker and transformed in DH5α cells (Invitrogen). Clones were selected and
tested by transient transfection to determine knockdown of mGRβ. After confirmation of knockdown the construct was co-transfected together with vectors expressing gag-pol, REV and VSV-G into 293FT cells (Invitrogen) to generate a third generation lentiviral construct. Transfection was achieved using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 100 ng total DNA per cm² of the growth plate or well. The supernatants were harvested and the cell debris was removed by centrifugation at 2000xg. The supernatant was used to infect mouse embryonic fibroblast (MEF) cells after addition of polybrene (5 ng/ml, Sigma Chemical Co., St. Louis, MO) to establish cell lines with stable down-regulation of mGRβ mRNA (mGRβ KD) or expressing empty vector. After 72 h the cells were sorted by flow cytometry for GFP by the Flow Cytometry Core Facility at the University of Toledo Health Science Campus. GFP positive cells were used for all experiments.

**Transfection and Reporter Assays.** Expression vector for mGRβ (pMGRβ-H57) was constructed as described above. The Ringold laboratory had already developed a plasmid for mouse GRα, pSV2Wrec (21). Both plasmids were transiently transfected into COS-7 cells (African green monkey kidney cells lacking an endogenous GR) and protein expression was measured via Western blotting. Dominant-negative activity was measured by luciferase assay using the GR-responsive minimal reporter pGRE₂EIB-Luc (54) and pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was
achieved using Lipofectamine 2000. Twenty-four hour post-transfected cells were treated with vehicle or 1 μM Dex or 1 μM RU486 for an additional 24 h until harvest. Cell lysates and assay were performed using the Promega luciferase assay system. Statistical analyses employed the Student’s t-test or ANOVA using GraphPad Prism v5.0a for Mac (GraphPad Software).

**Gel Electrophoresis and Western Blotting.** Whole cell extracts were prepared from COS-7 cells that were transiently transfected for 48 hours with either pMGRβ-H57 or pSV2Wrec using Lipofectamine 2000. Control cells were untransfected COS-7 cells that do not express GR. Protein content was determined by BCA method of Pierce. Protein samples were resolved by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 1 hour in TBS [TBS; 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 3% BSA. Subsequently, the membrane was incubated overnight at 4°C with FiGR antibody for total GR or rMGRβ antibody for mGRβ at a dilution of 1:1000 in TBS. After three washes in TBST (TBS plus 0.1% Tween 20), the membrane was incubated with an infrared anti-rabbit (IRDye 800, green) or anti-mouse (IRDye 680, red) secondary antibody labeled with IRDye infrared dye (LI-COR Biosciences) (1:15,000 dilution in TBS) for 2 hours at 4°C. Immunoreactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences).
**Immunoadsorption of GR Complexes.** Cells were harvested in HEMG (10 mM HEPES, 3 mM EDTA, 20 mM sodium molybdate, 10% glycerol, pH 7.4) plus protease inhibitor cocktail and set on ice for ten minutes followed by Dounce homogenization. Supernatants (cytosol) were collected proceeding a 10 minute 4°C centrifugation at 20,800 x g, then precleared with protein A or G-Sepharose nutating for 1 hour at 4°C. Samples were spun down, split into equal aliquots of cytosol, and immunoadsorbed overnight with FiGR antibodies against total GR, rMGRβ antibody for mGRβ, and appropriate controls (non-immune mouse IgG, or pre-immune rabbit serum) at 4°C under constant rotation. Pellets were washed 5-7 times with TEG (10 mM Tris, 3 mM EDTA, 10% glycerol, 50 mM NaCl, 20 mM sodium molybdate, pH 7.4) and complexes were eluted with 6XSDS sample buffer.

**Animals.** Adult, male C57/BL6 mice maintained on a normal diet *ad libitum*, or subjected to a fasting-refeeding regimen were used as tissue donors. Fasting encompassed 16 h (including the over-night 12 h dark cycle), followed by 8 h of refeeding *ad libitum* with normal chow at the start of the light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee of The University of Toledo.
ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants DK70127 (to E.R.S.) and DK54254 (to S.M.N.), and by a United States Department of Agriculture grant 38903-02315 (to S.M.N.). Terry Hinds, Jr. was supported by a predoctoral NIH National Research Service Award (F31DK84958). The authors thank Rudel Saunders and Qiong Wu for advice on plasmid construction.

Disclosure Statement: The authors have nothing to disclose.
References


receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. Mol Med 2:597-607


24. van der Vaart M, Schaaf MJ 2009 Naturally occurring C-terminal splice variants of nuclear receptors. Nucl Recept Signal 7:e007


53. **Kim SH, Kim DH, Lavender P, Seo JH, Kim YS, Park JS, Kwak SJ, Jee YK**


54. **Allgood VE, Oakley RH, Cidlowski JA** 1993 Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. J Biol Chem 268:20870-20876
Figure Legends

**FIG. 1.** Detection of Intron 8 Transcripts of the Mouse GR Gene. (A) Genomic organization (not to scale) of the mGR gene, showing location of forward primer (exon 7) and reverse primers (1-5) used for RT-PCR analysis (B). Predicted PCR product sizes for reverse primers 1-4 are indicated. Only primers in the intron 8 (I-8) region yielded products. In the case of primer 4, two products were predicted based on alternate utilization of a donor or acceptor site (Fig. 1S). Primers 4 and 5 (not shown) yielded no products on several attempts, consistent with published results (25). UTR, untranslated region. Con, amplification without template.

**FIG. 2.** Isolation and Sequencing of Full-length mGRβ mRNA. (A) Genomic organization (not to scale) of the mGR gene, showing location of forward primer (exon 1) and reverse primers (1 & 2) used for RT-PCR analysis (B). Primer 3 in conjunction with the forward primer in exon 1 was used to generate a second full-length mGRβ product that was cloned and sequenced to yield the organization of mGRβ mRNA is seen in (C). Primer 3 in conjunction with the forward primer in exon 2 was used to generate the cDNA cloned into pcDNA3.1+ (see Fig. 5). Con, amplification without template.
FIG. 3. Sequence and Functional Domain Comparisons of Mouse and Human GRβ. (A) Carboxyl-terminal regions of GRα and GRβ were aligned for each species. Overall percent homologies for entire proteins are indicated. Vertical lines indicate borders between exon 8 and distal domains. Light gray boxes indicate conservative substitutions. (B) The mGRβ isoform exhibits a functional domain structure that is nearly identical to hGRβ. Compared to mGRα, the β isoforms of both species have reduced and distinct C-terminal regions that lack the AF-2 domain (helix 12). These features account for their reduced ability to bind hormone and activate transcription. AF = activation function, DBD = DNA-binding domain, H = hinge region, LBD = ligand-binding domain, FiGR = epitope recognized by FiGR monoclonal antibody. HSP90 binding regions of mGRα and hGRβ are shown, along with putative site in mGRβ.

FIG. 4. Tissue Expression Profile of Mouse GR mRNA Isoforms. Real-time PCR analysis of tissues and MEF cells was performed with primers to intron 8 (mGRβ) or exon 9 (mGRα). All values were normalized to MEF cell mGRα and represent means +/- SEM for two independent samples/tissues. Adult, male C57/BL6 mice fed normal chow ad libitum were used as donors.

FIG. 5. Cloning, Western-blot and Indirect Immunofluorescence Analysis of mGRβ. (A) Primers spanning the ATG start site in exon 2 and the distal region of intron 8 (see Fig. 2) were used to isolate the full-length mGRβ cDNA, followed
by cloning into pcDNA3.1 to yield the pMGRβ-H57 vector. COS-7 cells were transfected with 8 µg each of pSV2Wrec (mGRα), pMGRβ-H57 (mGRβ) or empty vector (Mock), followed by Western-blotting with FiGR mouse monoclonal antibody that recognizes a common epitope on both mGR isoforms. (B) A rabbit polyclonal antibody specific to mGRβ (rMGRβ-Ab) was generated using the unique 15 amino acid terminal sequence. Whole cell extracts from COS-7 cells transfected with pSV2Wrec, pMGRβ-H57 or empty vector (Mock) were simultaneously probed with FiGR and rMGRβ-Ab antibodies. The Odyssey infrared detection system utilizing 680 nm (red) and 800 nm (green) emitting counter antibodies was used to detect FiGR (mGRα) and rMGRβ-Ab, respectively. Results show that the rMGRβ-Ab antibody reacts only with mGRβ, not with mGRα. (C) MEF cell lysates were immunoadsorbed with rMGRβ-Ab (I) or pre-immune (PI) serum, followed by blotting with rMGRβ-Ab, using enhanced chemiluminescence (ECL). Whole cell extracts from COS-7 cells transfected with pMGRβ-H57 were used for comparison. (D) Two separate MEF cell lysates were immunoadsorbed with FiGR (I) or non-immune (NI) IgG, followed by sequential blotting with rMGRβ-Ab and FiGR, using ECL. Relative densitometric ratios of mGRβ to mGRα are shown. (E) Indirect immunofluorescence using pre-immune serum, rMGRβ-Ab, or rMGRβ-Ab blocked with mGRβ peptide was performed on MEF cells treated or untreated with Dex (100 nM, 2h).
FIG. 6. Hormone Sensitivity and Dominant-Negative Activity of mGRβ. (A) COS-7 cells transfected with pSV2Wrec (mGRα) or pMGRβ-H57 (mGRβ) were assayed for luciferase activity at the pGRE2E1B-Luc reporter following treatment with Dex (1 µM), RU486 (1 µM), or vehicle control. Values were normalized to transfection efficiency (renilla) and represent means +/- SEM for three independent treatments. (B) Luciferase (pGRE2E1B-Luc) activity in COS-7 cells was measured following transfection with mGRα and increasing amounts of mGRβ and treatment with 100 nM Dex (S.E.M., N=3, **p<0.01 vs Dex, mGRα alone). (C) To assess dominant-negative activity of mGRβ at endogenous genes, MEF cells expressing mGRα were transfected with pMGRβ-H57 or control vector and treated with or without Dex (100 nM), followed by real-time PCR analysis. Values represent means +/- SEM of three independent treatments assayed in triplicate (**p<0.001, *p<0.05 vs Dex, mGRα alone).

FIG. 7. Gene Silencing of mGRβ. Lentiviral delivery of mGRβ shRNA was used to make a MEF cell line with stable down-regulation of mGRβ. A control cell line was infected with lentivirus expressing empty vector. (A) Complete mRNA constructs for mGRα or mGRβ were amplified via PCR to show mGRβ knockdown. (B) mGRβ shRNA and vector MEF cells were treated with 100 nM Dex for 2 h or vehicle, followed by real-time PCR analysis. Values represent means +/- SEM of three independent treatments assayed in triplicate (**p<0.01, *p<0.05 vs Dex, Vector Control).
FIG. 8. Hormonal and Dietary Control of mGRβ Expression. Real-time PCR analysis of mGR isoforms in (A) MEF cells treated with 100 nM Dex (***p<0.001), (B) RAW 264.7 monocytic macrophage cells treated with 10 nM TNFα (**p<0.01), (C) MEF cells treated with 100 nM insulin (***p<0.001), and (D) livers of adult male C57/BL6 mice subjected to fasting-refeeding (**p=0.01). All values were normalized to 18S RNA and represent means +/-SEM of three independent treatments assayed in triplicate.
FIGURE 1.
FIGURE 2.
FIGURE 3.
FIGURE 4.
FIGURE 5.
FIGURE 6.
FIGURE 7.
FIGURE 8.
SUPPLEMENTAL FIGURE 1S.

Calculations for Putative GR-beta Arising from Exon 8 or Intron 8

A

No splice site predictions for Exon 8.

Donor site predictions of mGRbeta in intron 8.

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Accepter site predictions of mGRbeta in intron 8.

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B

Primer 1: 3'TAAGGACATCKGCACACCC 5'
Primer 2: 3'CTGTCTTTGGCCTTTTGAGATAGG 5'
Primer 3: 3'CTTTGCGCTTTTTGAGATAGGATC 5'

Red = branch point sequences
Blue = polypyrrimidine tract

C

Intron 8

gtaagttacaagaaataatacagaaaacacacagcaaaaaa
agaaaaagttgggtgggatggtcttcgcttttaaattcacaagacaacagcaagtttagagcttaattggagttctgctactaatagctagcggcagaccaggc
agcagagatacaagatagcttatttttttaaacacatagattactttttgtttttttctcctcggcatggaggagaagtttaagtgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
SUPPLEMENTAL FIGURE 2S.

Calculations for Putative GR-beta Arising from Exon 9

| Donor site predictions for mGR beta in Exon 9 | 1187 | 1201 | 0.62 | exon16,splicing | 1890 | 1904 | 0.82 | AAGACAGTGGTGCCTC 32bp product |
| Acceptor site predictions for mGR beta in Exon 9 | 2189 | 2203 | 0.58 | gcaaatctgtaaacatctttgAt | 2242 | 2276 | 0.70 | ttctccagagagat |

| Primer 4: 3'-CTAGGAGAGAGAGCTCTGAG 5' |
| Primer 5: 3'-CTCTAGCTTGGAGAGAGAG 5' |

Predicted PCR product sizes of GR beta from Introns 9 or Exon 9

Exon 748 = 28bp

mGR Exon 9

| mGR Exon 9 (Primer4): | 2839 | 32bp product |
| AC Acceptor 1 (highest score): 289 + 124 = 413bp product |
| AG Acceptor 2 (lowest score): 289 + 235 = 524bp product |

Green = mGRalpha mRNA Exon 9 sequence

Blue = Highest scores for splice-site predictions
SUPPLEMENTAL FIGURE 3S.

Mouse GRbeta mRNA sequence

1 atggactctca aagaaactctt aagtcctccct cgtgagacag aagtcctcca cagttgtcct
d1 gcgcggggga ggggaaagct gatggaatct tataaaacct ctaaggggtaa agctcaagtc
121 aaggttctct cgctctccag ctcaagctgt gcgctgttct aagccagatt caaccaagca
d1 agatgtccess tttgtatccg aaaaaggtca gcaagaaatg cacaagctaa gcaagaaatg
241 caccaacccg gacgcaagtatt atcacaagcccc gtttcctgtg ccatagggtaa gttatgggaa
d1 gagaagagaaa ccaagaaatg ggggaaagct tggggctacc cacaaggtg gcaagaaatg
361 ctcttctcttg cggagaagca tttggctgctt ctggaagaag gcatgccaac ccctcaatag
d1 tcagaaaaagga gataaagaaa ctcaacccctc gcggcggggt gcgcggggga ggggagagat
481 acaagagaag aagtttccca gactcaactct gataaactctt cagcaagacta aaattagaad
541 agccagactcg gcaaacaagcc ggggggtgtg gtaatttaa taaacaagaa acagaaactct
601 gacatcttcag agagagggaa gttttttctgcc gggttcctccag gttaaagaagc aacgtagaggt
661 ccttgaggtgt cagacctgtt gatagatgaa agctcttgctt cttttttggc gggagagat
712 gagatactcc tttgcagaaag gacagctgtat gaggatgctag acaattttta ggaattgctag
781 actaaactcctt aaaaaactgag taactggagat cacaattttcat caagccccccag atgtggtgga
841 ctgcccccaag ttgaaacaca gtaaattgag ttcattagca tttcgaccac tgggtgttatt
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1021 caatactgaa tggataacag atctctacttt gctgtagcag attcagagcc agytttttat
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1141 gacaaactcag ttttctcttg ggctctagaa ttcgaagagg cgtgcaggttt tttcatttagg
1201 tattcagcag ctggaagatag acctagattg agtttcccttg cgcccagctc ctcctacagca
1260 aagggcagcc cttctcaaatc ctgctcttttg gctgctggaat aaggttggtg gatgcatatt
1321 ggggtgtctga cgtgtaagga ctgtaaatttc ttctttatag gaggctgtgg aagacagac
1381 aattcattttt cctgggtggaag aatagttgct acattatgtaa attcagagaa aaaaaattct
1441 ccagctatgc gcgtactcggaa atgtctcccc agggtggtgaacctgacagc gcggaaaaac
1501 aagaaaaatat tttaaagctg tcaacgagcg acctcgaggt ctccaaacag cacatttat
1561 aacgctacta cacaattatg ttcctgcccc gcgtcccaacag ttgctgctatg ccgctgctat
1621 cttggagcg tggctcagcc tggagcattt tattgcatatt aagtcatctgc gttctccagac
1681 tcagcagagga aaatttataca aacaatttac gcgggtgagct atgtttagtg gcgcgggaatt tagtggcagc
1741 gtgaataggg caagggccat aacaagattc agaaacttaa cacoattgag gtaatctagcc
1801 ctctctactagg ttcctctcatct ttcctgttct cgcgtttttctc ctttccccag ttttttctgc
1861 caagcaagaga ggttatattg cttgactatg cctattttat gttataaaga gacaggaatg
1921 acetacacttt gctgttagct gatgataataa cacagtctgt ttttctccca gtagatttat
1981 agatggaggg atctctcttgag aggtaatca gttatgagat gttctctctct ttcctctctca
2041 gttctctagg aagttctggga ggccaggaag agtttgggtg cttgctctag acagtttagag
2101 aaagcgagta ggacaccccta gttctccaagc gcagggcggag cggcgggtgc ggtcagcggg
2161 ttcttcacca tggaaacactt ttgagccgcct atgtcaattg gtaattccag acaataattcc
2221 aaaaaaacaag caaaaaaaaa aagtag
Chapter 3

Protein Phosphatase 5 Mediates Lipid Metabolism Through Reciprocal Control of Glucocorticoid and PPARγ Receptors

(Unpublished Manuscript)

Terry D. Hinds, Jr.¹, Lance A. Stechschulte¹, Harrison A. Cash¹, Donald Whisler¹
Weidong Yong², Michael J. Garabedian³, Weinian Shou² and Edwin R. Sanchez¹*

¹Center for Diabetes and Endocrine Research, Department of Physiology & Pharmacology, University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614, USA
²Herman B. Wells Center for Pediatric Research, Section of Pediatric Cardiology, Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, USA
³Department of Microbiology, New York University Cancer Institute, New York University School of Medicine, 550 First Avenue, New York, New York 10016, USA

Running title: PP5 & Adipogenesis

*Address requests to: Edwin R. Sanchez, (419) 383-4182, FAX (419) 383-2871
edwin.sanchez@utoledo.edu
Keywords: Protein phosphatase, TPR proteins, glucocorticoid receptor, peroxisome proliferator-activated receptor, insulin, insulin resistance, adipogenesis, obesity

SUMMARY

Glucocorticoid (GR) and peroxisome proliferator-activated receptor gamma (PPARγ) regulate adipogenesis by controlling the balance between lipolysis and lipogenesis. Here, we show that protein phosphatase 5 (PP5), a nuclear receptor co-chaperone, is a key regulator of adipogenesis. Elevated expression of PP5 was found in adipose of mice subjected to high fat diet and in 3T3-L1 and MEF cells subjected to adipogenic stimuli. Importantly, stimulated PP5KO MEF cells showed almost no lipid accumulation and high levels of fatty acids in the media that correlated with reduced expression of adipogenic markers (aP2, CD36, perilipin). These phenotypes were completely reversed in PP5KO cells following reintroduction of PP5. Loss of PP5 increased phosphorylation of GR at serines 212 and 234 and elevated dexamethasone (Dex)-induced activity at pro-lipolytic genes. In contrast, PPARγ in PP5KO cells was hyperphosphorylated at serine 112 but had reduced rosiglitazone-induced activity at lipogenic genes. This work identifies PP5 as a fulcrum point in nuclear receptor control of the lipolysis/lipogenesis equilibrium, and as a potential target in the treatment of obesity.
INTRODUCTION

In humans, obesity often results from alterations to carbohydrate and lipid metabolism, processes principally controlled by insulin, glucocorticoids and fatty acid signaling molecules. Metabolic disorders or high fat diets cause dysregulation of metabolism, resulting in lipid accumulation in adipose, muscle and liver, making these organs insensitive to insulin and other hormones. In times of stress or fasting, glucocorticoids (GCs) stimulate fat breakdown in adipose tissue and protein degradation in muscle to generate non-hexose substrates, such as amino acids and glycerol, for gluconeogenesis in the liver (Vegiopoulos and Herzig, 2007). Interestingly, chronic glucocorticoid elevation leads to insulin resistance and glucose intolerance, which may result in type II diabetes mellitus and metabolic syndrome. In adipose, the equilibrium between lipolysis and lipogenesis is controlled by two opposing nuclear receptor members: glucocorticoid receptor alpha (GRα) and proliferator-activated receptor gamma (PPARγ) (Lehrke and Lazar, 2005). GRα regulates the expression of lipolytic and anti-lipogenic genes, such as HSL, DGAT1, GILZ, ANGPTL4 and PDK4. Conversely, PPARγ upregulates the expression of genes, such as CD36, SREBP1c and perilipin, that promote lipid uptake, synthesis and storage (Spiegelman, 1998; Lefterova and Lazar, 2009; White and Stephens, 2009). Signaling molecules that can regulate the balance of actions of these hormones are important to this equilibrium.
The Hsp90 chaperone complex plays an essential role in control of GRα and other nuclear receptors (Pratt and Toft, 1997; Smith and Toft, 2008). As co-chaperones to Hsp90, the tetratricopeptide repeat (TPR) proteins FK506-binding protein 52 (FKBP52), FKBP51 and protein phosphatase 5 (PP5) are important modulators of GR activity (Smith, 2004; Davies and Sanchez, 2005; Hinds and Sanchez, 2008). Hormone-free GRα in most cells preferentially interacts FKBP51 and PP5, with reduced presence by FKBP52 (Banerjee et al., 2008). In spite of this, FKBP52 is still needed as a positive regulator of GRα, controlling its transcriptional activity in a gene-specific manner (Wolf et al., 2009), while most studies suggest that FKBP51 and PP5 are negative regulators of GR (Zuo et al., 1999; Reynolds et al., 1999; Denny et al., 2000). In contrast to GRα, very little is known concerning Hsp90 and TPR protein involvement with PPARs. Studies by Vanden Heuvel and Perdew showed interaction of Hsp90 with PPARα, PPARβ and PPARγ (Sumanasekera et al., 2003b; Sumanasekera et al., 2003a). In the case of PPARα, Hsp90 was inhibitory of receptor transcriptional activity. Of particular interest, use of a peptide specific to the TPR domain of PP5 caused a large increase in PPARα activity (Sumanasekera et al., 2003a), providing the first, albeit indirect, evidence that PP5-mediated dephosphorylation is important to PPAR function.

GRα and PPARγ are differentially regulated by phosphorylation. In response to ligand, GR phosphorylation serines 212, 220 and 234 (mouse sequence) has been observed, with most studies showing a positive correlation
with GR activity (Bodwell et al., 1998; Ismaili and Garabedian, 2004). Phosphorylation of PPARγ is typically induced by growth factors which utilize MAPK signaling to target serine 112 and inhibit the differentiation-inducing properties of the receptor (Hu et al., 1996; Adams et al., 1997). Dephosphorylation of GR by PP5 has been documented and, in general, the results support a model in which PP5 serves to attenuate GR transcriptional activity. Interestingly, no phosphatase has yet been discovered that specifically targets PPARγ.

PP5 is a unique member of the PPP family of serine/threonine phosphatases in that it contains TPR domains (Chinkers, 1994; Chen et al., 1994; Becker et al., 1994). Binding of PP5 to Hsp90 occurs through the TPR domains and is required to activate phosphatase activity. Interestingly, the TPR domains contain binding sites for long-chain, polyunsaturated fatty acids, binding of which also activate PP5 activity (Kang et al., 2001; Ramsey and Chinkers, 2002). We, therefore, reasoned that a major role of PP5 may be to regulate phosphorylation of signaling molecules, especially nuclear receptors, involved in lipid metabolism. In this work, we address this hypothesis by testing the role of PP5 in models of adipogenesis controlled by GRα and PPARγ. We show that PP5 can directly regulate the phosphorylation states of GRα and PPARγ to control the balance of lipolysis and lipogenesis.
EXPERIMENTAL PROCEDURES

Materials. Dexamethasone, HEPES, DMEM powdered medium, Tris, EDTA, PBS, sodium molybdate, protease inhibitor cocktail, non-immune mouse IgG2A, and sodium chloride were all obtained from Sigma (St. Louis, MO). Iron supplemented newborn calf serum was from Hyclone Laboratories Inc. (Logan, UT). Immobilon-FL polyvinylidenefluoride membrane was obtained from Millipore Corporation (Bedford, MA). Lifofectamine 2000 transfection reagent and OPTI-MEM were obtained from Invitrogen Corporation (Carlsbad, CA).

Cell Lines and Culture. Mouse embryonic fibroblasts (MEF) were isolated from wild-type (WT) and PP5 knockout (KO) E13.5 embryos. Cells were cultured in DMEM with 15% FBS until confluence. Fibroblasts were the only cells that attached and proliferated. Immortalized MEFs were generated by transfecting primary cells with vector for SV40-large T antigen. Transformed cells maintain normal MEF morphology with high proliferative activity. MEF cells were routinely cultured and maintained in Dulbecco’s Modified Eagles’s Medium (DMEM) containing 10% iron-supplemented bovine calf serum with 1% penicillin-streptomycin.

Adipogenesis Assays.
Inductions of 3T3-L1 preadipocytes were differentiated with a cocktail of 170 nM insulin, 100 µM isobutylmethylxanthine (IBMX), and 1 µM glucocorticoid
treatment. WT and PP5 KO MEF cells were differentiated using 830 nM insulin, 100 µM isobutylmethylxanthine (IBMX), 5 µM rosiglitazone and 1 µM glucocorticoid treatment. Cells were cultured in DMEM with 10% FBS until confluence. Cells were routinely cultured and maintained in DMEM containing 10% fetal bovine serum with 1% pencillin-streptomycin. Upon differentiation cells were treated with Nile Red to analyze lipid development. After images were taken of the cells the total RNA was extracted to be used for Real-time PCR analysis. Quantification of free fatty acids in the media was performed by Wako NEFA C non-esterfied free fatty acid kit (Wako Chemicals USA, Inc., Richmond, VA), and measured on a Spectra Max Plus spectrophotometer (Molecular Devices).

**Transient Transfection.** For transient transfection cells were plated on a 6-well dish in DMEM containing 10% iron supplemented calf-serum pre-stripped of endogenous steroids by 1% (w/v) dextran-coated charcoal for 24 h prior to transfection and allowed to grow to 85-90% confluency for GR studies and serum free media for PPARgamma. Cells were washed with OPTI-MEM and transfected using Lipofectamine 2000, according manufacturer’s protocol. OPTI-MEM was removed after 5h and DMEM containing dextran-charcoal stripped serum was added. All hormone treatments were done 24-48 h posttransfection for 2 h.
**Transfection and Reporter Assays.** WT and PP5KO cells that were transiently transfected for 48 hours with either pSV2Wrec (GRalpha), PPARgamma2 pcDNA 3.1 and RXR alpha using Lipofectamine 2000. Plasmids were transiently transfected into WT and PP5 KO MEFs and activity was measured by luciferase assay using the GR-responsive minimal reporters pGRE2EIB-Luc (54) or PPRE-luc and pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was achieved using Lipofectamine 2000. Twenty-four hour post-transfected cells were treated with vehicle or 1 µM Dex or 1 µM rosiglitazone for an additional 24 h until harvest. Cell lysates and assay were performed using the Promega luciferase assay system. Statistical analyses employed the Student’s t-test or ANOVA using GraphPad Prism v5.0a for Mac (GraphPad Software).

**Whole Cell Extraction.** Cells were washed and collected in 1X PBS followed by centrifugation at 1500 X g for 10 min. The supernatant was discarded and the pellet was re-suspended in 1X PBS. After a short spin at 20,800 X g for 5 min at 4°C the pellet was rapidly frozen on dry ice ethanol mix and stored at -80°C for 30 min. The frozen pellet was then re-suspended in 3 volumes of cold whole cell extract buffer (20mM HEPES, 25% glycerol, 0.42M NaCl, 0.2mM EDTA, pH 7.4) with protease inhibitors and incubated on ice for 10 min. The samples were centrifuged at 100,000 X g for 5 min at 4°C. Protein levels were measured spectrophotometrically by a Nanodrop 2000 (Thermo fisher Scientific,
Wilmington, DE). The supernatants were either stored at -80°C or used immediately for Western analysis to determine protein expression levels.

**Gel Electrophoresis and Western Blotting.** Whole cell extracts were prepared from WT and PP5KO cells. Protein samples were resolved by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 1 hour in TBS [TBS; 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 3% BSA. Subsequently, the membrane was incubated overnight at 4°C with primary antibody. FiGR monoclonal antibody against GR and rabbit polyclonal antibody against PP5 were generous gifts from Jack Bodwell (Dartmouth Medical School, Hanover, NH) and Michael Chinkers (University of South Alabama, College of Medicine, Mobile, AL), respectively. Phospho GR S212, S220 and S234 antibodies were a gift from Michael Garabedian (New York University, School of Medicine, New York, NY). Antibodies against anti-Green Fluorescent Protein (GFP) mouse monoclonal IgG (sc-9996), PPARgamma (sc-7273) and HSP90 (sc-8262) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). S112 Phospho-PPARgamma2 antibody was purchased from Abcam (Abcam PLC, Cambridge, MA). Anti-flag M2 monoclonal IgG (F-3165) were all obtained from Sigma (St. Louis, MO). Primary antibodies were diluted to 1:1000 in TBS and incubated at 4°C overnight. After three washes in TBST (TBS plus 0.1% Tween 20), the membrane was incubated with an infrared anti-rabbit (IRDye 800, green) or anti-
mouse (IRDye 680, red) secondary antibody labeled with IRDye infrared dye (LI-COR Biosciences) (1:15,000 dilution in TBS) for 2 hours at 4°C. Immunoreactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences).

Green Fluorescent Protein Imaging. WT and PP5 KO MEF cells were seeded on laminin-coated cover slips in 60mm dishes at 300,000 to 500,000 cells per dish. Cells were maintained in medium containing charcoal-stripped serum before fluorescence imaging. The cells were transfected 48 h later with either GFP-tagged GR and PPARgamma2 constructs or empty vector (pEGFP-C1). Fluorescent images of the living cells were obtained 24 h post-transfection and 1 h after vehicle or hormone treatment using an Olympus IX70 inverted microscope equipped with a Leica DMIRE2 confocal microscope (Leica, Mannheim, Germany). Cells were scanned at low laser power to avoid photobleaching. Leica confocal software was used for data analysis. The figures show representative cells from each type of transfection. At least 50-100 cells from each transfection were inspected.

Quantitative Real-Tme PCR Analysis. Total RNA was extracted from mouse tissues using 5-Prime PerfectPure RNA Tissue Kit (Fisher Scientific Company, LLC). cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). PCR amplification of the cDNA was performed by quantitative real-time PCR using
qPCR Core kit for SYBR Green I (Applied Biosystems). The thermocycling protocol consisted of 10 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 61°C, and 20 sec at 72°C and finished with a melting curve ranging from 60–95°C to allow distinction of specific products. Primers were designed specifically to each gene using Primer Express 3.0 software (Applied Biosystems). Normalization was performed in separate reactions with primers to 18S mRNA (TTCGAACGTCTGCCCTATCAA and ATGGTAGGCACGGCGACTA). To study whether genomic sequences were amplified, a control sample was used in which no cDNA added.

Animals. Adult, male C57/BL6 mice maintained on a normal diet ad libitum, or subjected to a fasting-refeeding regimen were used as tissue donors. Fasting encompassed 16 h (including the over-night 12 h dark cycle), followed by 8 h of refeeding ad libitum with normal chow at the start of the light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee of The University of Toledo.

Diet and metabolic analyses
The breeding colony was maintained on standard chow [regular diet (RD)] containing 12 kcal% fat ad libitum. Experimental mice were separated into two groups of 8-wk-old males. One group was fed (ad libitum) a RD for 4 wk, and the second was fed (ad libitum) a HF diet containing 45 kcal% fat (catalog no.
To eliminate the confounding effects of fasting on GC responses, all tissues and blood samples were collected from animals under random-fed conditions. The HF regimen chosen is known to induce borderline to moderate steatosis and insulin resistance in most strains of mice, without stimulating hepatic inflammation.

Whole venous blood was drawn from retro-orbital sinuses. Blood glucose levels were measured using a glucometer (Accu-check Aviva; Roche Diagnostics, Indianapolis, IN). Corticosterone (MP Biomedicals, Irvine, CA), plasma insulin, and C-peptide levels (Linco Research, St. Charles, MO) were measured by RIA. Plasma FFAs (Wako Bioproducts, Richmond, VA) and TGs (Pointe Scientific, Canton, MI) were measured by colorimetric assay.

**Statistical Analysis**

Data were analyzed with Prism 5 (GraphPad Software, Inc.) using unpaired t-tests. P values less than 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

**PP5 Expression Is Increased During Adipogenesis**

As a first test of the role of PP5 in lipid metabolism, expression levels of PP5 were measured by quantitative real-time PCR (qRT-PCR) in the metabolic organs of adult, male C57B6 strain mice subjected to a 30-day high-fat diet. In response to this regimen, the animals exhibited increased body weight, visceral adiposity,
hyperglycemia and other standards symptoms (unpublished observations) of diet-induced metabolic syndrome. Interestingly, PP5 mRNA levels were significantly elevated in abdominal white adipose tissue in response to the high fat diet, but were normal in liver and muscle (Figure 1A), suggesting that PP5 in adipose is particularly sensitive to diet. To corroborate this result, NIH 3T3-L1 preadipocytes were used to determine if PP5 expression increased during in vitro adipogenesis. Differentiation was induced with the standard Dex, insulin and IBMX (DII) cocktail typically used on 3T3-L1 cells (Wolins et al., 2006). The results (Figure 1B) show treatment-induced expression of PPARγ and hormone-sensitive lipase (HSL) – two key makers of adipocyte differentiation (Langin et al., 2005; Deng et al., 2006). Differentiation also increased the levels of PP5, FKBP51, and GRβ, while leaving two other nuclear receptor co-chaperones, FKBP52 and Cyp40, unchanged. PP5 and FKBP51 are well-known negative regulators of GR, while the recently discovered mouse GRβ isoform appears to act as a dominant-negative inhibitor of mGRα (Hinds et al., 2010). For this reasons, it seemed likely that attenuation of GR activity might be an essential aspect of the adipogenesis process. Moreover, PP5 can be directly bound and activated by fatty acids, such as arachidonic (Kang et al., 2001; Ramsey and Chinkers, 2002). We therefore reasoned that PP5 may be functioning as a fatty acid-induced signaling molecule in adipose tissue, perhaps targeting GR and other nuclear receptors.
PP5 Deficiency Blocks Adipogenesis

We have previously described the generation of mice with global ablation of PP5 and isolation of wild-type and PP5-ablated (KO) MEF cells (Yong et al., 2007). Robust induction of adipogenesis in the MEF cells was achieved by treatment with DII cocktail with the addition of rosiglitazone to activate PPARγ, as previously described for similar MEF cell lines (Rangwala et al., 2003). Figure 2 shows a strong lipogenic response in WT cells, as measured by Nile Red staining and direct biochemical assay. In contrast, PP5-KO cells had significantly decreased lipid accumulation. Lack of induction was not an artifact of the PP5-KO cell line, as rescue of PP5 expression in the KO line completely reversed the phenotype. Additionally, differentiated PP5-KO cells had significantly higher free fatty acids in the serum compared to differentiated WT cells (Figure 2C), suggesting a defect of reduced lipid import, elevated export, or both.

To investigate the underlying cause, qRT-PCR was performed on RNA extracted from the same set of differentiated and undifferentiated MEF cells. The data show elevated expression of six adipogenic markers in treated WT cells (Figure 3). Interestingly, PP5-KO cells showed reduced expression of all six markers – a pattern that was completely reversed in the PP5 rescued cells. In PP5-KO cells the strongest repression was observed for the fatty acid binding protein aP2, the lipid storing protein perilipin and the fatty acid importer CD36. Less robust repression was seen for hormone-sensitive lipase (HSL) and the glucose transporters Glut-1 and -4. This pattern suggests that the principal net
effect of PP5 loss may be to disrupt lipid uptake and storage, although as yet undiscovered lipolytic and anti-lipogenic processes may also contribute.

**Loss of PP5 Increases the Anti-Lipogenic Actions of Glucocorticoids**

Because there are reports that PP5 acts as a negative regulator of GR, we reasoned that elevated anti-lipogenic activity of GR may contribute to the inability of PP5-KO cells to accumulate lipid. In our system, GR activation could occur as the result of the single treatment with Dex on day 1 of induction or through endogenous steroids in the serum or produced in the cell. As a first step, we excluded the day 1 treatment with Dex from the adipose-inducing regimen. As seen in Figure 4A, exclusion of Dex from the cocktail did not block lipid accumulation in WT cells and did not reverse the lipid-lean phenotype in PP5-KO cells. Yet, GCs are clearly anti-lipogenic, as continuous treatment with Dex during adipogenic induction greatly reduced lipid buildup in both WT and KO cells (Figure 4B). Taken as whole, these results suggest that elevated GR activity may contribute to the lipid-resistant state of PP5-KO cells, but that other factors, most likely PPARγ, must also be involved.

**Reciprocal Control of GR and PPARγ by PP5**

To directly test the roles of PP5 on GR and PPARγ, transcriptional activities of each receptor were measured in WT and PP5-KO MEF cells. In initial experiments, MEF cells were transfected with reporter genes (Figure 5). The
results obtained demonstrated elevated Dex-induced GR activity at the MMTV-luciferase construct in the KO cells, but greatly reduced basal and rosiglitazone-induced PPARγ activity using a PPRE-luciferase reporter in the same cells. Analysis of receptor levels by Western-blotting (Figure 5) showed reduced levels of GR in the PP5-KO cells (see also Figure 2), but higher levels of PPARγ in the KO cells (see also Figure 10). Taken together, the results show that loss of PP5 augments GR activity but diminishes PPARγ, in spite of opposite effects on receptor protein levels. Importantly, the results suggest that PP5 may regulate the balance of lipolysis and lipogenesis by reciprocally modulating the activities of the receptors.

As a more relevant test, qRT-PCR analysis was performed to determine the role of PP5 on endogenous metabolic genes controlled by each receptor. Figure 6 shows results of WT and PP5KO MEF cells treated with Dex. GR activity was greater in the KO cells at all genes tested, except for FKBP51, demonstrating that PP5 control of GR is gene-specific. Of particular relevance are the results for glucocorticoid-induced leucine zipper protein (GILZ), pyruvate dehydrogenase kinase-4 (PDK4) and CD36. GILZ was recently shown to inhibit adipogenesis in mesenchymal cells (Shi et al., 2003). PDK4 is a metabolic gating protein that promotes gluconeogenesis by preventing the shunting of pyruvate to de novo lipid synthesis (Sugden and Holness, 2003). The CD36 results demonstrate that PP5 loss can also increase GR-mediated gene repression. Taken as a whole, the
results demonstrate that PP5 serves to inhibit several anti-lipogenic properties of GR.

In contrast to dexamethasone treatment, PP5-KO MEF cells treated with rosiglitazone showed significantly reduced expression of some, but not all, PPARγ regulated genes (Figure 7). Most notable was CD36 which showed dramatic reductions in both basal and ligand-induced expression, similar that seen with the PPRE promoter (Figure 5). Reduced basal expression most likely reflects the ability of PP5 to control the ligand-independent activity of PPARγ, or PPARγ that is activated by endogenous or serum-derived fatty acids. Indeed, phosphorylation of human PPARγ has been shown to inhibit both its ligand-dependent and independent activities (Adams et al., 1997). Because CD36 is repressed by GR but induced by PPARγ, the reciprocal nature of PP5 regulation on these receptors may result in near-maximal repression of its expression in the PP5-KO cells. This alone may be the single greatest contributor to reduced lipid content observed in the PP5-KO MEF cells (Figure 2). However, other PPARγ regulated genes were affected in the KO cells, such as PEPCK, which promotes glyceroneogenesis in adipose (Tontonoz et al., 1995), and HES1 a known repressor of lipogenic gene expression (Herzig et al., 2003). Curiously, repression of HES1 by PPARγ in the WT cells was converted to activation in the KO cells, suggesting that PP5 may differentially regulate the transactivation versus transrepression properties of the receptor at some genes. In any case, the high level of HES1 in KO cells is also consistent with the lipid-lean phenotype of these
cells. Like GR, PP5 regulation of PPARγ is also gene specific, as no change in SREBP1c expression was seen in the absence of PP5.

**Localization and Hormone-induced Translocation of GR and PPARγ Are Not Affected by PP5**

Hormone-induced nuclear translocation is an integral step in the activation of most nuclear receptors. In the case of GR, circumstantial evidence suggests that PP5 may regulate this process, as it can be bound by dynein, the motor protein of microtubule-based transport (Galigniana et al., 2002). Although only limited evidence exists for PPARγ interaction with PP5 (Sumanasekera et al., 2003a), its cellular localization could be directly or indirectly controlled by PP5. As an initial test of this hypothesis, the localization of PP5 in MEF cells was determined by indirect immunofluorescence (Figure 8A). Consistent with observations in other cell lines (Chen et al., 1994; Banerjee et al., 2008), the majority of PP5 was perinuclear, suggesting a potential role in nuclear transport. The localization of receptors was determined by transfecting WT and PP5-KO MEF cells with GR-GFP and PPARγ-GFP expression constructs. GR-GFP was localized to the cytoplasm under basal conditions and translocated to the nucleus with Dex treatment in both WT and PP5-KO cells (Figure 8B). GR-GFP imaging was consistent with fractionation analysis (data not shown). In contrast to GR, inactive PPARγ-GFP was primarily localized to the nucleus, although a small fraction was observed in the cytoplasm. Treatment with rosiglitazone had no
effect on distribution in WT and PP5-KO cells. These results demonstrate that PP5 does not prevent or promote hormone-induced nuclear translocation of GR or PPARγ, suggesting that altered activities result from intrinsic functions of the promoter-occupied receptor, most likely due to changes in phosphorylation.

**PP5 Regulates the Phosphorlyation States of GR and PPARγ**

To directly test whether PP5 controls the phosphorylation states of GR and PPARγ, Western blot analyses were performed with phospho-serine antibodies specific to each receptor. In the case of GR, hormone-activation is known to cause elevated phosphorylation at serines 212, 220 and 234 (mouse sequence) in the AF-1 region of GR (Wang et al., 2007; Zhang et al., 2009). Some studies report a positive correlation between phosphorylation status and hormone-induced transcriptional activity of the GR (Zuo et al., 1998; Zuo et al., 1999). In Figure 9, GRs from WT and PP5-KO cells treated with Dex were compared for phosphorylation status at serines 212, 220 and 234. In WT cells, Dex-induced phosphorylation was significantly increased at serines 220 and 234. In PP5-KO cells, hyperphosphorylation was observed at all three residues, with the largest increases at serines 212 and 234. No increase of basal GR phosphorylation in PP5KO cells was observed. These results are strong evidence that GR is a direct target of PP5 phosphatase activity and that the primary role of PP5 is to attenuate GR action by preventing hyperphosphorylation. These observations are in good agreement with studies by Honkanen using anti-sense knockdown of PP5 which
demonstrated increased transcriptional activity of GR and increased GC-mediated growth arrest (Zuo et al., 1999; Dean et al., 2001). Studies by Goleva and Garabedian using PP5 siRNA also found elevated phosphorylation of GR. In each case, however, GR activity was reduced rather than increased (Wang et al., 2007; Zhang et al., 2009). It should be noted that the Garabedian study showed reduced GR activity at three genes not tested in our study and no effect on a fourth, GILZ, which we find to be upregulated with PP5 loss (Figure 6). Thus, it is likely that the PP5 exerts gene-specific control on GR and other nuclear receptors.

Like GR, PPARγ is also a phosphoprotein. However, most studies show that phosphorylation of PPARγ, especially by mitogen-activated protein kinase pathways, inhibits its activity (Lazar, 2005; Burns and Vanden Heuvel, 2007). The principal target of mitogen signaling is serine 112 of mouse PPARγ (homologous to S114 in humans) and a S112A mutation results in receptor with elevated basal and ligand-induced activities (Camp and Tafuri, 1997; Camp et al., 1999). To determine whether PP5 dephosphorylates S112, WT and PP5-KO cells were transfected with wild-type PPARγ and the S112A mutant. Western-blot analysis with an antibody against S112 showed significantly increased phosphorylation at S112 for untreated and rosiglitazone-bound PPARγ (Figure 10). A trend to increased S112 phosphorylation was also seen in PP5-KO cells treated with tetradecanoyl phorbol acetate (TPA) to activate phosphorylation cascades. Curiously, levels of total PPARγ protein were consistently higher in the PP5-KO
cells, as already seen in Figure 5, even though activity is considerably reduced. Thus, it is likely that PP5-mediated dephosphorylation of PPARγ may also control proteolytic processing of the receptor. More importantly, our results identify PP5 as the first *bone fide* phosphatase to act on PPARγ. At this late date, this observation is somewhat surprising. MAP kinase phosphatase-1 (MKP-1) has been shown to dephosphorylate PPARα (Vanden Heuvel et al., 2003), and rosiglitazone-activated PPARγ can upregulate MKP-1 expression (Jan et al., 2009). To the best of our knowledge, MKP-1 actions on PPARγ have yet to be reported. Since phosphorylation inhibits PPARγ but has a positive effect on PPARα (Shalev et al., 1996), it is possible that MKP-1 upregulation is the mechanism by which PPARγ antagonizes the actions of PPARα. With this in mind, it will be interesting to see whether PP5 also targets PPARα to decrease its activity.

In summary, we have identified protein phosphatase 5 as a reciprocal modulator of GR and PPARγ that serves to antagonize the lipolytic/anti-lipogenic actions of GR, while simultaneously promoting the adipogenic actions of PPARγ (Figure 11). PP5 achieves this by selectively dephosphorylating GR at serines 212 and 234, causing inhibition of GR transcriptional activity at genes such as CD36 and PDK4. PP5 dephosphorylates PPARγ at serine 112, promoting its transcriptional activity at the pro-lipogenic genes CD36, aP2 and others. Thus, PP5 can be considered a pro-lipogenic phosphatase, making it an new candidate for the treatment of obesity. PP5 is also an attractive drug target, as it is activated
by polyunsaturated but not saturated fatty acids, suggesting that development of an inhibitory fatty acid analog is feasible.
ACKNOWLEDGEMENTS

The authors are indebted to Dr. Rudel Saunders for help in developing the \textit{in vitro} adipogenesis assay.

This work was supported in part by National Institutes of Health grants DK70127 and DK73402. T.D.H, Jr. was supported by a predoctoral NIH National Research Service Award (F31DK84958).

Disclosure Statement: The authors have nothing to disclose.
REFERENCES


FIGURE LEGENDS

Figure 1. PP5 Expression Is Increased During Adipogenesis.

(A) Quantitative real-time PCR analysis of PP5 expression in abdominal white adipose tissue, skeletal muscle and liver in C57Bl6 male mice fed regular (RD) or high fat diet (HFD) for 30 days. *P < 0.05, ± S.E.M, N = 8.

(B) qRT-PCR analysis of 3T3-L1 cells on day 0 (d0) or day 12 (d12) of adipogenic induction using DII cocktail. ***P < 0.001, ± S.E.M, N = 9.

[Statistical key for all figures: * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Same pattern applies to # and ^ symbols.]

Figure 2. Absence of PP5 Blocks Lipid Accumulation During Adipogenesis.

(A) Western-blot analysis of whole cell extracts from WT and PP5-KO MEF cells demonstrating complete lack of PP5 in the KO cells and restoration of PP5 expression following rescue of PP5-KO cells with Flag-PP5 (KO-R). Note reduced levels of GR in KO cells that is also rescued to wild-type levels in the KO-R cells. Hsp90 was used as loading control.

(B) Detection of lipid accumulation in WT, PP5-KO and KO-R cells by Nile Red staining. Adipogenic differentiation (D) or undifferentiation (U) was achieved by treatment of MEF cells with DII cocktail plus rosiglitazone.

(C) Biochemical quantification of intracellular lipid in cells treated in (B). ### P < 0.001 (vs WT U), ***P < 0.001 (vs WT D), ^^^P < 0.001 (vs PP5-KO D), ± S.E.M, N = 3.
(D) Biochemical quantification of free fatty acid content in the growth media of cells treated in (B). ## P < 0.01 (vs WT U), **P < 0.01 (vs WT D), #P < 0.05 (vs WT D), ± S.E.M, N = 3.

Figure 3. PP5 is Required for Induction of Adipogenic Markers.
qRT-PCR analysis of WT, PP5-KO and KO-R cells following differentiation (D) or undifferentiation (U) with DII plus rosiglitazone. # = vs WT-U , * = vs WT-D, ^ = vs KO-D, ± S.E.M, N = 6 - 9.

Figure 4. Role of Dexamethasone in MEF Cell Adipogenesis.
(A) WT and PP5KO MEF cells were treated with adipogenic cocktail containing (+Dex) or not containing Dex (−D) on the first day of treatment, or with cocktail containing Dex on each day of treatment (+Dex ET). Lipid accumulation detected by Nile Red staining.

(B) Direct biochemical measurement of intracellular lipid content in cells of panel A. * = vs WT same condition, # = vs WT +Dex, ^ = vs WT Dex ET, ± S.E.M, N = 3.

Figure 5. Reciprocal Regulation of GRα and PPARγ Activity by PP5.
(A) WT and PP5KO MEF cells expressing endogenous GR (Western blot shown) were assayed for Dex-induced GR activity at the MMTV-luciferase reporter.
(B) WT and PP5KO MEF cells were transfected with PPARγ expression vector (Western blot shown) and assayed for rosiglitazone-induced PPARγ activity at the PPRE-luciferase reporter.

Figure 6. Absence of PP5 Increases GRα Activity at Select Endogenous Metabolic Genes.

Undifferentiated WT and PP5KO MEF cells were treated with or without Dex followed by qRT-PCR analysis of the indicated genes. # = WT vs WT, * = KO vs WT, ^ = KO vs KO, ± S.E.M, N = 9.

Figure 7. Absence of PP5 Decreases PPARγ Activity at Select Endogenous Metabolic Genes.

Undifferentiated WT and PP5KO MEF cells were transfected with PPARγ and treated with or without rosiglitazone followed by qRT-PCR analysis of the indicated genes. # = WT vs WT, * = KO vs WT, ^ = KO vs KO, ± S.E.M, N = 9.

Figure 8. PP5 Does Not Regulate Intracellular Trafficking of GRα and PPARγ.

(A) Localization of PP5 in WT and PP5KO MEF cells by indirect immunofluorescence.

(B) Localization of GRα-GFP in WT and PP5KO cells treated with or without Dex.
(C) Localization of PPARγ-GFP in WT and PP5KO cells treated with or without rosiglitazone.

**Figure 9. PP5 Control of GRα Phosphorylation at Serines 212 and 234.**

(A) Whole cell extracts of WT and PP5KO MEF cells treated with or without Dex were analyzed by Western-blotting with antibodies specific to serines 212, 220 and 234 of GRα. FiGR antibody was used to detect total GRα.

(B) Quantitation of GR bands seen in panel A. # = WT vs WT, * = KO vs WT, ^ = KO vs KO, ± S.E.M, N = 4.

**Figure 10. PP5 Control of PPARγ Phosphorylation at Serine 112.**

(A) Whole cell extracts of WT and PP5KO MEF cells transfected with wild-type PPARγ or S112A mutant were analyzed by Western-blotting with antibody specific to serine 112, or antibody against total PPARγ. Prior to harvest cells were untreated (C) or treated with TPA or rosiglitazone.

(B) Quantitation of PPARγ bands seen in panel A. Phospho PPARγ signals were normalized to total PPARγ at each condition. # = WT vs WT, * = KO vs WT, ± S.E.M, N = 4.

**Figure 11. PP5 is a Fulcrum of the Lipogenesis-Lipolysis Equilibrium by Reciprocally Modulating GRα and PPARγ.**
PP5 is reciprocal modulator of GR and PPARγ that antagonizes the anti-lipogenic actions of GR, while simultaneously promoting the lipogenic actions of PPARγ. PP5 achieves this by selectively dephosphorylating GR at serines 212 and 234, causing inhibition of GR transcriptional activity at genes such as CD36 and PDK4. PP5 dephosphorylates PPARγ at serine 112, promoting its transcriptional activity at the pro-lipogenic genes, such as CD36, aP2.
FIGURE 1.
FIGURE 2.

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C

![Graph](image25.png)

D

![Graph](image26.png)
FIGURE 3.
FIGURE 4.

A

+ Dex

WT  PP5KO

− Dex

WT  PP5KO

+ Dex ET

WT  PP5KO

B

Lipid Accumulation

WT  PP5KO

+Dex  +Dex  −Dex  −Dex  Dex ET  Dex ET

***  ***  #  #  ***  ***
FIGURE 5.
FIGURE 8.
FIGURE 9.
FIGURE 10.

A

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B

- WT
- PP5KO

Graph showing the comparison of pS112 PARγ/Total PARγ between WT and PP5KO groups. The graph includes error bars and statistical symbols (#, **, *) indicating significance levels.
FIGURE 11.
Chapter 4

Contribution of Glucocorticoid Receptor β to
Glucocorticoid Resistance in Adipose
(Unpublished Manuscript)

Terry D. Hinds, Jr., Lance A. Stechschulte, Jasmine Lawrence and Edwin R. Sanchez*

Center for Diabetes & Endocrine Research (CeDER)

and the Department of Physiology & Pharmacology,

University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614,
USA.

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*Corresponding author: Department of Physiology & Pharmacology, University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614-5804, (419) 383-4182, FAX (419) 383-2871, edwin.sanchez@utoledo.edu.

Abbreviated title: Glucocorticoid Receptor β and Adipose

Keywords: glucocorticoid receptor beta, mouse, metabolism, obesity, inflammation, steroids, nuclear receptor

This work was supported in part by National Institutes of Health grants DK70127 (to E.R.S.) and DK54254 (to S.M.N.), and by a United States Department of Agriculture grant 38903-02315 (to S.M.N.). Terry Hinds, Jr. was supported by a predoctoral NIH National Research Service Award (F31DK84958).

Disclosure Statement: The authors have nothing to disclose.
Abstract

Glucocorticoids (GCs) are hormones that are thought to play a role in obesity. The enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) which can increase intracellular concentrations of GCs, has been shown with overexpression to exacerbate obesity. The role that the glucocorticoid receptor (GR) has in regulating obesity has been shown to be lipolytic in acute treatments, however, long-term treatment results in obesity and the metabolic syndrome. We are interested in the switch from lipolytic to lipogenic actions of GCs. The GR gene in both humans and mice is alternatively spliced to produce two isoforms, GRα and GRβ. The GRα is the hormone binding isoform that regulates gene responses to GCs. GRβ, on the other hand, does not bind GCs and is inhibitory to GRα. In this study, we show that mice on a high fat diet increased GRβ in liver and adipose, but had no change in GRα expression. Additionally, we show in 3T3-L1 preadipocyte cells that mGRβ expression is increased upon maturation into adipocytes and that this correlates with reduced responsiveness to GCs. This suggests that obesity is a possible state of GC resistance that may be due to elevated GRβ expression.
INTRODUCTION

The onset of obesity in the United States is startling. The development of obesity is complex and the mechanisms are not fully understood. Obesity can lead to insulin resistance, diabetes, heart disease, fatty liver and inflammation of the liver and adipose tissues. Discovery of the preadipocyte 3T3-L1 mouse cell [1] line has aided in understanding the factors that contribute to development of adipocytes and eventually discovery of the adipocyte master controller, peroxisome proliferated activated receptor γ (PPARγ) [2]. Several drugs, such as the thiazolidinedione rosiglitazone, have been developed as PPARγ agonists that are used in treatment of type II diabetes mellitus [2]. Stimulation of preadipocytes into mature adipocytes can be performed by treatment of insulin, glucocorticoids (GCs) and isobutylmethylxanthine (IBMX) [3]. The actions of GCs have been shown to increase the expression of PPARγ and CCAAT/enhancer-binding protein β (C/EBPβ) that leads to adipocyte maturation [3]. Diseases such as Cushing Syndrome, obesity, type 2 diabetes, cardiovascular disease and the metabolic syndrome have been linked to the actions of GCs on the body [4]. However, GCs have also been documented to induce lipolysis in adipocytes, especially during times of fasting and exercise [5]. Interestingly, an enzyme, 11β hydroxysteroid dehydrogenase type 1 (11β HSD1), which converts cortisone to cortisol, has been shown to significantly increase expression in mature adipocytes [6]. Mice that had adipocyte specific overexpression of 11β HSD1 showed significant weight gain and was thought to
be because of increased intracellular cortisol to stimulate adipocyte hypertrophy [7]. This is controversial, however, because 11β HSD1 can also oxygenate cholesterol to produce oxysterols that are liver x receptor (LXR) α agonist [8]. LXRα is well known to induce lipogenesis and adipocyte hypertrophy [9] [10].

The actions of GCs have been shown to be lipolytic in nature, and inhibition of GR may result in adipocyte hypertrophy. Overexpression of the co-chaperone protein, FK506-binding protein 52 (FKBP52) results in increased GR activity [11]. We have generated FKBP52-deficient mice, which are viable at birth and apparently normal into adulthood, except for infertility [12] [13]. Yet, cells derived from FKBP52 KO mice have reduced GR activity [14]. Our FKBP52 heterozygous mice, when fed a high fat diet, acquire symptoms similar to the metabolic syndrome; in which they develop hyperglycemia, hyperlipidemia, hyperinsulinemia and weight gain [15]. In comparison to the 11β HSD1 model of increased GCs that stimulate adipocyte hypertrophy, the FKBP52 heterozygous mice have reduced GR activity and develop the metabolic syndrome phenotype. This implies that reduced GR activity and possibly GC resistance may be a major cause of the development of obesity.

The discovery of the mouse GRβ isoform that is inhibitory to the GC binding isoform, GRα, has been recently reported [16]. The study found increased expression of mGRβ after insulin and dexamethasone treatment of cells. GRα mRNA, on the other hand, decreased after GC treatment. This implies that decrease of the GRα or FKBP52 and increased expression of GRβ may result
in reduced GC activity in adipocytes. With this in mind, we speculated that GRβ may be involved in regulating adipocyte hypertrophy and the overall development of obesity. In this study, we measured adipocyte responsiveness to GCs in 3T3-L1 preadipocyte cells that were undifferentiated and differentiated. Treatment with GCs showed that GR has significantly less activity up maturation into adipocytes. We analyzed the expression of GRα and GRβ after adipocyte maturation, and results show that GRβ significantly increases and GRα was unchanged. Manifestation of obesity and adipocyte hypertrophy may be states of GC resistance due to elevated GRβ expression.

MATERIALS AND METHODS

Materials. Dexamethasone, HEPES, DMEM powdered medium, Tris, EDTA, PBS, and sodium chloride were all obtained from Sigma (St. Louis, MO). Iron supplemented newborn calf serum was from Hyclone Laboratories Inc. (Logan, UT). Lipofectamine 2000 transfection reagent and OPTI-MEM were obtained from Invitrogen Corporation (Carlsbad, CA).

Cell Lines and Culture. 3T3-L1 preadipocytes were differentiated with a cocktail of 170 nM insulin, 100 µM isobutylmethylxanthine (IBMX), and 1 µM glucocorticoid treatment. Cells were cultured in DMEM with 10% FBS until confluence. Cells were routinely cultured and maintained in Dulbecco’s Modified Eagles’s Medium (DMEM) containing 10% fetal bovine serum with 1%
pencillin-streptomycin.

**Quantitative Real-Tme PCR Analysis.** Total RNA was extracted from mouse tissues using 5-Prime PerfectPure RNA Tissue Kit (Fisher Scientific Company, LLC). Total RNA from MEF cells was extracted as described above. cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). PCR amplification of the cDNA was performed by quantitative real-time PCR using qPCR Core kit for SYBR Green I (Applied Biosystems). The thermocycling protocol consisted of 10 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 61°C, and 20 sec at 72°C and finished with a melting curve ranging from 60–95°C to allow distinction of specific products. Primers were designed using Primer Express 3.0 software (Applied Biosystems). A common forward primer in exon 8 (AAAGAGCTAGGAAAAGCCATTGTC) was used in conjunction with a reverse primer in intron 8 for mGRβ (CTGTCTTTGGCTTTTGAGATAGG), or a reverse primer in exon 9 for mGRα (TCAGCTAACATCTCTGGGAATTCA) and TNFα primers (F – GACTCAAATGGGCTTTCCGA and R – TCCAGCCTCATCTGAGACAGAG) Normalization was performed in separate reactions with primers to 18S mRNA (TTGAACGCTGCCCCTATCAA and ATGGTAGGCACGGCGACTA). To study whether genomic sequences were amplified, a control sample was used in which no reverse transcriptase was added (non-RT control).
**Animals.** Adult, male C57/BL6 mice maintained on a normal diet ad libitum, or subjected to a fasting-refeeding regimen were used as tissue donors. Fasting encompassed 16 h (including the over-night 12 h dark cycle), followed by 8 h of refeeding ad libitum with normal chow at the start of the light cycle. All procedures were approved by the Institutional Animal Care and Utilization Committee of The University of Toledo.

**Diet and metabolic analyses**

The breeding colony was maintained on standard chow [regular diet (RD)] containing 12 kcal% fat ad libitum. Experimental mice were separated into two groups of 8-wk-old males. One group was fed (ad libitum) a RD for 4 wk, and the second was fed (ad libitum) a HF diet containing 45 kcal% fat (catalog no. D12451; Research Diets, New Brunswick, NJ). To eliminate the confounding effects of fasting on GC responses, all tissues and blood samples were collected from animals under random-fed conditions. The HF regimen chosen is known to induce borderline to moderate steatosis and insulin resistance in most strains of mice, without stimulating hepatic inflammation.

Whole venous blood was drawn from retro-orbital sinuses. Blood glucose levels were measured using a glucometer (Accu-check Aviva; Roche Diagnostics, Indianapolis, IN). Corticosterone (MP Biomedical, Irvine, CA), plasma insulin, and C-peptide levels (Linco Research, St. Charles, MO) were measured by RIA. Plasma FFAs (Wako Bioproducts, Richmond, VA) and TGs (Pointe Scientific,
Canton, MI) were measured by colorimetric assay.

RESULTS

Glucocorticoid Resistance After Adipose Differentiation

In this assay, 3T3-L1 preadipocyte cells were induced to undergo adipocyte differentiation by treatment with insulin, IBMX, and dexamethasone. Preadipocyte cells were induced for 12 days to mature into adipocytes. Upon differentiation, mature adipocytes in hormone-free media were treated with 100 nM dexamethasone and compared to preadipocytes treated as a control group. Figure 1 shows that adipocyte marker genes aP2, CD36, LXRα, perilipin and adiponectin went up as expected upon differentiation into adipocytes; dexamethasone significantly decreased mRNA expression of these genes. Interestingly, GR activated genes: SGK, IKKβ, IkBβ, IkBα and DUSP1 were significantly reduced in adipocytes. These suggest that upon maturation of adipocytes that development of GC resistance occurs. We wanted to further analyze the possible causes of the reduced GR activity and analyzed expression of an isoform of GR, GRβ, which is a known inhibitor to the GC binding isoform GRα.

GRβ mRNA Expression Significantly Increases in Adipocyte Differentiation of 3T3-L1 cells.

Upon maturation in adipocytes, total RNA was extracted and converted to
cDNA and this was used for Real-time PCR analysis to determine the expression of GRα, GRβ and TNFα. The results show that expression of mGRβ significantly increased, while no increase of GRα, and a significant decrease of TNFα. Typically, mammals on a high fat diet (HFD) become inflamed in adipose and liver. Therefore, we wanted to determine the expression profile of TNFα, GRα and GRβ in tissues of mice on a HFD.

**GRβ Expression Increases in Adipose and Liver of Mice on a High Fat Diet**

The mice in this study were fed HFDs for 4 weeks and the triglyceride, insulin and glucose plasma levels were measured, and these mice developed insulin resistance and inflammation of the liver (data not shown). At the end of the study, muscle, liver and visceral tissues were collected and total RNA was extracted. Real-time PCR results show that GRα expression is unchanged on a HFD in all tissues analyzed (Figure 3). GRβ and TNFα, however, significantly increased in liver and adipose of mice on a HFD.

**DISCUSSION**

In this work, evidence is provided that GC resistance due to elevated expression of GRβ is a contributing factor to development of obesity and associated inflammation. To date, GC resistance, via GRβ appears to occur in many immunological diseases and drug-resistant states, such as asthma [17], leukemia [18]; ulcerative colitis [19], chronic sinusitis [20], and systemic lupus
erythematous [21]. Patients suffering from these diseases can be refractory to GC treatment. Not surprisingly, increased activation of pro-inflammatory factors and elevated hGRβ expression has been noted in cases of GC resistance. The discovery of the mouse GRβ isoform has provided a mammalian model to test GC resistance.

Only one study outside the immune system has been published on hGRβ. It showed that exposure of skeletal muscle to GCs leads to a decline in GRα expression and a concomitant increase of GRβ [22]. Because skeletal muscle is critical for glucose uptake in response to insulin and because GRα mediates the insulin antagonist actions of GCs in this organ [23], this is the first indirect evidence that GRβ upregulation may be an important mechanism for maintaining organ sensitivity to insulin. In a previous study, we showed that the mouse GRβ significantly increases in cells upon treatment of insulin and in livers of mice that were fasted and refed [16]. This indicated a possible role of GRβ in sensitizing tissues and cells to insulin. We tested mouse 3T3-L1 preadipocyte cells by differentiating them into adipocytes and treating with the synthetic GC, dexamethasone. The results indicate that there is a significant decrease in the ability of GR to respond to GCs, compared to its actions in preadipocytes. Figure 2 demonstrates an increase of GRβ in these cells and no increase of GRα. This implies that the GC resistance may be directly correlated to the actions of GRβ in adipocytes.
Mammals on a high fat diet eventually develop inflammation of liver and adipose. GRβ may be decreasing the ability of GRα to function as an anti-inflammatory and as a result increased cytokine and inflammation occurs with ascending levels of GRβ. Proinflammatory cytokines, such as TNFα and IL-1, increase expression of hGRβ via the NF-kB pathway [24]. Mouse GRβ was also shown to increase expression upon treatment of TNFα on murine immune cells [16]. Mice that were randomly fed or on high fat diets showed no change in GRα expression, but do show significant increases of GRβ and TNFα in liver and adipose. This suggests that GRβ may be working to increase inflammation and GC resistance and as a result lipid accumulates in these tissues.

Obesity is thought to be due to actions of GCs on adipose, even though these patients have normal levels of GCs. Increased local amplification of cortisol by 11β HSD1 is controversial; however, inhibitors have been shown useful in reducing plasma insulin, glucose and total cholesterol [25]. Our FKBP52 heterozygous mice, with reduced GR activity, showed the importance of GR in regulating the actions of obesity. This demonstrates that the actions of GR are lipolytic and reducing the ability of GR to respond to GCs results in adipocyte hypertrophy. Additionally, inflammation of the adipose in mammals may cause severe decreases in GR activity, as NF-kB is a known inhibitor to GCs. 3T3-L1 cells do not develop inflammation, but GR activity was significantly reduced at GR regulated genes (Figure 1). Inflammation of adipose results in increased expression of adipokines and immune cell invasion that results in insulin
resistance and increased GRβ expression. Thus, obesity may be the result of elevated GRβ and GC resistance.
REFERENCES


Figure Legends

Figure 1. Glucocorticoid resistance in 3T3-L1 adipocytes upon maturation.
Media was switched to hormone-free media the day before treatment of undifferentiated and differentiated cells. Treatment of 100nM dexamethasone for 2 h showed that in mature adipocytes that GR responds less to hormone compared to preadipocytes. ***P < 0.001, ± S.E.M, N = 3.

[Statistical key for all figures: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.]

Figure 2. GRβ expression significantly increases in adipocytes. Total RNA was extracted from 3T3-L1 cells that were undifferentiated and differentiated. Real-time PCR results show that GRα is unchanged, but a significant increase of GRβ expression. ***P < 0.001, ± S.E.M, N = 3.

[Statistical key for all figures: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.]

Figure 3. Mice on a high fat diet have significantly higher expression of GRβ.
Mice on a high fat diet for 4 weeks were used to extract total RNA from muscle, liver and adipose. The liver and adipose had significant increases of TNFα and GRβ, while muscle showed no difference. GRα mRNA expression was unchanged in all tissues. ***P < 0.001, ± S.E.M, N = 3.

[Statistical key for all figures: * = P < 0.05, ** = P < 0.01, *** = P < 0.001.]
FIGURE 1.
FIGURE 2.
FIGURE 3.

A. GR alpha & beta in adipose on HFD

B. GR alpha & beta in liver on HFD

C. GR alpha & beta in muscle on HFD
Chapter 5

Summary, Discussion and Conclusions

This dissertation explores the hypothesis that glucocorticoid (GC) resistance is an underlying cause of obesity and associated inflammation. The development of GC resistance can occur in different manners as was observed in Chapter 2 with GRβ, and Chapter 3 with PP5. Increased expression of FKBP51 and decrease of FKBP52 also result in GC resistance, as was observed in New World monkeys. Reducing the actions of GR to respond to GCs results in inflammation and reduced lipolysis, as was seen in the FKBP52 heterozygous mice on high fat diet. Immune system homeostasis is balanced by GCs, which regulate immune cell turnover by suppressing cytokine production and promoting apoptosis. GC insensitivity due to elevated GRβ expression results in higher levels of proinflammatory cytokines, leading to escalated cell growth and reduced cell death [79].

As well, proinflammatory cytokines, such as TNFα and IL-1, increase expression of GRβ via the NF-κB pathway [80]. Cytokine production is increased in asthma patients, most of whom respond to GC treatment. However, some subjects that do not benefit from GC therapy because of elevated GRβ [81]. Fatal
asthma has been linked to extremely high levels of GRβ expression in both large and small airways of these subjects [82]. Ascending levels of GRβ in asthma patients results in many disease complications, as well as GC resistance that can verge to a complete loss of drug response [83]. Other inflammatory diseases linked to high levels of GRβ include: ulcerative colitis [84], ankylosing spondylitis [85]; cigarette smoking [86]; leukemia [87]; and systemic lupus erythematosus [88].

Only one study outside the immune system has been published on hGRβ. It showed that exposure of skeletal muscle to GCs leads to a decline in GRα expression and a concomitant increase of GRβ [89]. Because skeletal muscle is critical for glucose uptake in response to insulin and because GRα mediates the insulin antagonist actions of GCs in this organ [90], this is the first indirect evidence that GRβ upregulation may be an important mechanism for maintaining organ sensitivity to insulin. Chapter 2 shows the discovery of the much needed mouse model of GRβ that fully support this hypothesis, and that GRβ may assist in sensitizing tissues to insulin. This was observed in mice that were fasted and refed, GRβ significantly increased at time point 4-7 hours, which may function as a protective means to insulin responsiveness.

The actions of GCs are to work as anti-inflammatories and in Cushing’s syndrome patients with high circulating cortisol results in symptoms similar to the metabolic syndrome and inflammation of liver and adipose. We have shown that GCs increase expression of GRβ and FKBP51 and this may result in GC
resistance in Cushing’s patients, as was shown with the reduced ability of GR to bind ligand in these patients. High cortisol levels may result in a constant systemic elevation of GRβ and FKBP51 that result in increased cytokines and inflammation which will further worsen the subject.

Obesity is thought to be due to actions of GCs on adipose, even though these patients have normal levels of GCs. Increased local amplification of cortisol by 11β HSD1 is controversial; however, inhibitors have been shown useful in reducing plasma insulin, glucose and total cholesterol. Without H6PDH, 11β HSD1 loses its ability to reduce cortisone to cortisol and the oxidation-reduction reaction reverses to oxygenate cholesterol to produce oxysterols. These are agonist to LXRα that have been shown to exacerbate adipocyte hypertrophy. With the loss of reducing compatibility, 11β HSD1 oxidizes cortisol to cortisone that are agonist for MR. Knock down of MR in 3T3-L1 preadipocytes demonstrated MR’s ability to increase adipogenesis; knocked down of GR in the same study showed no difference in adipogenesis and lipid development. Actions of GR in adipogenesis, as an end result, are to increase expression of C/EPBβ and PPArγ that regulate lipogenesis. However, GCs are not needed in the differentiation cocktail as was observed in Fig 4A of Chapter 3. Removal of GCs had no effect on differentiation, however, adding dexamethasone to every media change resulted in significantly less lipids (Fig 4A, Chapter 3). This demonstrates that the actions of GR are lipolytic and reducing the ability of GR to respond to GCs results in adipocyte hypertrophy. Additionally, inflammation
of the adipose in mammals may cause severe decreases in GR activity. 3T3-L1 cells do not develop inflammation, but GR activity was significantly reduced at GR regulated genes (Fig 1, Chapter 4). Inflammation of adipose results in increased expression of adipokines and immune cell invasion that results in insulin resistance and increased GRβ expression that may also result in GC resistance. Thus, obesity may be the result of both insulin and GC resistance.

The GR works to control the lipolytic/lipogenic cycle in adipose, and inhibition of this results in adipocyte hypertrophy. Increased expression PP5, FKBP51 and GRβ and decrease of FKBP52 all may contribute to GC resistance in this tissue. FKBP52 heterozygous mice develop the metabolic syndrome with a high fat diet, which is interesting because the actions of GR have long been thought to increase obesity, as was the case with adipocyte specific overexpression of 11β HSD1. Sensitivity to GCs, as was observed in the FKBP51 knockout mice on a high fat diet was shown to be critical for resistance of diet induced obesity. The role that GRβ has in regulating obesity are to be elucidated, but may work to increase insulin sensitivity. Chapter 4 demonstrates that GRβ did increase in liver and adipose of mice on a high fat diet and may play a major role in regulating obesity. Increased GRβ expression may be the first step in adipocyte hypertrophy of mammals, and this may serve to sensitize to insulin and cause GC resistance and reduce lipolysis and eventually inflammation.

PP5 is also inhibitory to the actions of GR and was shown to be increased only in adipose of mice on a high fat diet. The actions of PP5 to dephosphorylate
GR and PPARγ gives this phosphatase a unique ability to promote lipogenesis over lipolysis. In Chapter 3, PP5 was shown to be critical to PPARγ gene regulated activity, and inhibitory to GR. This may decrease GR activity and sensitize PPARγ to store more lipids in adipose, which supports our hypothesis of GC resistance in adipose. The loss of PP5 resulted in significantly less lipids compared to wild-type cells. PP5 KO cells have significantly higher GR activity which may unbalance the lipolytic/lipogentic cycle to result in reduced transport of fatty acids to be stored during lipogenesis, as was observed with these cells having higher free fatty acids in the media after adipose differentiation (Fig 2D, Chapter 3).

GC resistance of the immune system of humans has been shown in many disease states. Whether this is through GRβ, FKBP51 or PP5 has to be elucidated, either way these will serve as great potential drug targets. The ability to increase lipolysis, as was seen in the FKBP51 KO mice and PP5 KO cells, is critical to regulation of these diseases. These targets are tissue specific in nature because of their differential expression. It may be uncovered that obese patients have high levels of one but not the other, and in these cases specific treatment can incur. This provides avenues for specific treatments of the cause of the obesity and will aid in providing treatment for these patients. The discovery of GRβ in mice and the development of the FKBP51, FKBP52 and PP5 knockout mice will allow us to better understand the role that these proteins have in regulating metabolism and
inflammation. With this in mind, obesity and the metabolic syndrome are states of GC resistance.
References


