The role of glucocorticoid receptor signaling in metabolic disease: a matter of time and sex

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5

General discussion and perspective

GENERAL DISCUSSION

This thesis focused on the complex role of glucocorticoid signaling in metabolic diseases, emphasizing novel insights into sex-specific responses, circadian influences, and therapeutic interventions. Glucocorticoid signaling plays a pivotal role in the regulation of various physiological processes. However, long lasting or excess exposure to glucocorticoids may lead to a range of metabolic side effects including hyperglycemia, insulin resistance, obesity, muscle loss and osteoporosis (1-4). In fact, very recent data indicate a degree of hypercortisolemia in over 20% of difficult-to-treat type 2 diabetes patients (5). Understanding the role of the glucocorticoid receptor (GR) in metabolic disease is also essential for improving the management of patients receiving chronic glucocorticoid therapy, as these individuals are at increased risk for developing metabolic complications. Therefore, elucidating the mechanisms by which the GR regulates metabolic homeostasis, and how these mechanisms are altered in various disease states, remains a crucial area of research in endocrinology.

Summary of the findings

In **chapter 2**, we investigated the effects of treatment with the synthetic glucocorticoid betamethasone and excess exposure to the endogenous glucocorticoid corticosterone on muscle function and atrophy in both male and female mice. We directly compared male and female mice, allowing us to identify sex differences in the glucocorticoid response in muscle. Corticosterone treatment led to reduced grip strength specifically in female mice, while muscle mass being decreased in both sexes. By performing RNA-sequencing, we observed that male mice exhibited more pronounced transcriptional responses to corticosterone as compared to female mice. We thus found stronger functional consequences in female mice, but more transcriptomic effects in male mice. The sex-difference following a synthetic glucocorticoid treatment regimen were somewhat different: we found that betamethasone administration reduced grip strength in both sexes, but that female mice were more sensitive to glucocorticoid-induced muscle atrophy. In an attempt to understand the sexually dimorphic glucocorticoid effects, we addressed the contribution of androgen signaling in male mice and found that part of the glucocorticoid responses in skeletal muscle were influenced by androgen deprivation. This finding did not suggest that glucocorticoid-induced muscle atrophy is completely androgen-dependent, as both sexes experienced atrophy and androgen signaling might only partly contribute to the differences.

In addition to sex differences in the glucocorticoid response, we found that the time of glucocorticoid administration influences its adverse effects. We describe the comparison of morning versus evening betamethasone administration in **chapter 3**. Morning (out of phase) betamethasone treatment significantly reduced insulin sensitivity and caused more potent effects on glucose

metabolism, compared to evening administration. We additionally found that the outcome of glucocorticoid treatment was dependent on the time of measurement. In general, circadian rhythm should thus be taken into account in research on glucocorticoids.

In **chapter 4**, we explored the role of glucocorticoid signaling in mouse model of androgen-induced polycystic ovary syndrome (PCOS). We observed that treatment with a GR antagonist only had limited effects for most of the metabolic features associated with PCOS/elevated androgen exposure. Nevertheless, we found that GR antagonism during the development of metabolic symptoms can result in improved glucose metabolism, with no strong effects on other DHT-exposed features including lipid metabolism.

Sex-Specific Responses to Glucocorticoid Treatment and Therapeutic Implications

Across all chapters, we observed consistent evidence of glucocorticoid-induced muscle atrophy, hyperglycemia, and insulin resistance. Given the widespread metabolic effects of glucocorticoid signaling, it is critically important to determine how these effects may differ between sexes, particularly those of muscle atrophy and metabolic outcomes described in **chapter 2**. We generally found that glucocorticoid treatment promotes muscle atrophy in male and female mice, but some glucocorticoid effects on muscle were sex-dependent. Male mice displayed a more extensive transcriptional response, including the upregulation of key atrogenes such as *Klf15*, *MurF-1* (*Trim63*), and *atrogin-1* (*Fbxo32*). Interestingly, despite the stronger atrophic gene response in males, functional impairment including grip strength was more pronounced in females. This discrepancy may indicate the involvement of additional, possibly non-genomic, pathways in the sex-specific responses to glucocorticoids. Crosstalk of glucocorticoid signaling with androgen signaling was previously described in the liver and other tissues (6), and may thus also play an important role in skeletal muscle function. The dominant androgen testosterone plays a dual role in muscle metabolism. On the one hand, it has anabolic effects, promoting muscle protein synthesis (7,8), but it also mitigates glucocorticoid-induced muscle atrophy. Androgens may not be able to fully prevent the activation of catabolic pathways including the upregulation of *Murf-1* and *atrogin-1* under conditions of high glucocorticoid exposure (9). In addition, prolonged dexamethasone treatment leads to significant decrease of androgen receptor (AR) mRNA expression in skeletal muscle and plasma androgen levels (10), which potentially further reduces the inhibitory effect of androgens on glucocorticoid-induced muscle atrophy.

To further explore the role of androgens in glucocorticoid-induced muscle atrophy, we chemically castrated male mice using degarelix, a GnRH antagonist that has several advantages over other castration procedures. GnRH agonists

initially evoke a surge of LH and FSH (and thus testosterone) before leading to a downregulation of GnRH receptors. Degarelix directly and irreversibly blocks the GnRH receptors without this initial surge (11,12). Androgen depletion alone already led to significant muscle atrophy and reduced grip strength. When combined with betamethasone treatment, an additive effect on muscle atrophy was observed, in particular on the gastrocnemius, EDL, and TA muscles. This points toward a protective role of androgens in muscle maintenance through their actions, which are likely counteractive against the glucocorticoid-driven catabolic processes. The activation of the Akt/mTOR pathway critical for muscle protein synthesis can be reduced with androgen deprivation (7). Synthetic glucocorticoids further downregulate this pathway, leading to more pronounced anabolic processes (13). These muscle changes were accompanied by a shift in the composition of fiber types, increasing the proportion of type 2A fibers at the expense of type 2B fibers. This shift is consistent with previous findings that glucocorticoid treatment can drive a transition from fast-twitch glycolytic fibers to more oxidative fiber types, potentially contributing to the observed reductions in muscle strength (14). This fiber type shift upon betamethasone treatment was less pronounced in female mice, but it should be noted that females have more slow-twitch (type 1) fibers than males (15). These fibers are oxidative and more resistant to fatigue and are generally less prone to atrophy under stress. Under prolonged glucocorticoid treatment regimens, the oxidative capacity of such slow-twitch fibers could become compromised, leading to muscle wasting (16).

Given the crucial role of androgens in muscle function and other metabolic processes, we also investigated the interplay between glucocorticoid and androgen signaling in the metabolic features of PCOS. Many of the clinical features of PCOS including insulin resistance and adiposity overlap with those observed in conditions of excess glucocorticoid exposure such as Cushing's syndrome (17,18). This overlap suggests that GR signaling may be an important modulator of PCOS-associated metabolic symptoms. The availability of a selective GR antagonist without affinity for the androgen and progesterone receptors allowed us to test this hypothesis (19). In **chapter 4**, we demonstrate that prolonged DHT exposure upregulates GR signaling machinery, particularly in key metabolic tissues like the liver and adipose tissue. Specifically, DHT increased the expression of GR mRNA and the enzyme 11β-HSD1 in the liver and gWAT. This is supported by earlier studies indicating that androgen signaling can modulate glucocorticoid metabolism, with DHT enhancing local glucocorticoid concentrations by activating 11β-HSD1 (6). This suggests that in PCOS, androgens may enhance local glucocorticoid activation, thereby amplifying glucocorticoid-mediated metabolic disturbances. These findings are consistent with the previous report showing that androgen exposure enhances the concentrations of corticosterone in the liver and adipose tissues (20). The increased expression of 11β-HSD1 may further stimulate the GR signaling, exacerbating insulin resistance and fat deposition. This mechanistic insight highlights the androgen-glucocorticoid interplay in the pathogenesis of PCOS

and underlines the potential of targeting GR signaling as a therapeutic strategy (21).

The estrogen receptor (ER) plays a critical role in maintaining metabolic and reproductive functions particularly in females. Estrogens typically exert protective effects against metabolic dysfunction through the regulation of glucose homeostasis by enhancing insulin sensitivity in the adipose tissue and skeletal muscle (22). Despite the potent effect of estrogens on maintenance of muscle mass, females are more sensitive to disuse atrophy (as a consequence of muscle inactivity) (23,24). Estrogen supplementation or activation of ER was shown to mitigate atrophy in male mice (25,26) but not in female rats (27). Besides, the lower levels of androgens in females could render them more sensitive to glucocorticoid-induced atrophy as the protective effects of androgens are attenuated. This discrepancy indicates that aromatase could play a role in maintaining muscle mass and reducing atrophy. Studies performed in ArKO mice reveal that muscle mass is significantly reduced and mice are more vulnerable to muscular atrophy in the absence of estrogens (28). This highlights the importance of estrogen signaling in male muscle maintenance. Moreover, it is likely that testosterone acts to maintain muscle protein synthesis and muscle function through its conversion into estradiol via aromatization and subsequent activation of ERs (29,30). Estrogen signaling can alleviate the degradation of type 1 fibers (31,32), although it may not be sufficient to fully prevent glucocorticoid-induced atrophy. In contrast, androgens have more pronounced effect on type 2 fibers, promoting muscle hypertrophy and strength (33). In PCOS, this could shift the balance due to the excess of androgen toward dampening beneficial effects from ER signaling and further promoting metabolic dysregulation via the dominant AR and GR pathways (34).

Therapeutic strategies targeting glucocorticoid signaling may hold potential in conditions like PCOS, given the upregulation of GR machinery by excess androgen exposure. In **chapter 4**, we show that preventive GR antagonism (during disease progression) improves glucose metabolism in DHT-exposed mice, indicating that early intervention by GR blockade can mitigate the metabolic consequences of androgen excess. Similarly, GR antagonism with RU486 was able to mitigate high-fructose-induced insulin resistance and lipid accumulation both in adipose tissues and liver (35). However, GR antagonism does not always result in improved metabolic outcomes, and despite the clear advantage of improving insulin sensitivity, the overall metabolic benefit of GR antagonism may be highly dependent on the disease model and the timing of intervention (36). This is illustrated by the fact that while preventive GR antagonism showed clear benefits in our study, GR antagonism that started after the onset of metabolic dysfunction did not yield similar improvements. It is possible that GR signaling mainly plays a role in the development of metabolic disturbances, or that compensatory pathways such as enhanced androgen signaling may dominate the metabolic dysregulation. One study found that androgens can modulate GR activity in adipose tissue and the liver, enhancing

insulin resistance and exacerbating fat accumulation in male mice (37). This suggests that androgen signaling can aggravate GR-mediated metabolic dysfunction. Moreover, once metabolic consequences are fully established, the compensatory mechanisms including heightened androgen signaling and mitochondrial dysfunction might minimize the influence of GR signaling (38,39). Thus, early intervention targeting GR signaling might be more effective in preventing the progression of hyperandrogenism-driven metabolic complications (39).

We observed a differential impact of GR antagonism on body composition and lipid metabolism. Compared with control mice, DHT exposure increased fat mass as well as lean mass. Preventive GR antagonism significantly decreased body weight and lean mass in mice by comparison with vehicle or DHT treatment alone. This was distinct from other models of metabolic disease in which GR antagonism resulted in reduced fat mass and improved lipid metabolism (40). However, in DHT-treated mice we found that neither preventive nor therapeutic GR antagonism altered the increased fat mass or adipose tissue weight. It is known that AR agonism can amplify GR transcriptional responses in white and brown adipose tissue, while AR antagonism attenuates these effects (6). This suggests that the role of GR antagonism on adiposity is blunted under conditions of hyperandrogenism and thus points out a complex interplay of glucocorticoid-androgen signaling in regulating adipose tissue function. Interestingly, GR antagonism enhanced triglyceride-derived fatty acid uptake in adipose tissues of control mice but not in DHT-treated mice. This blunted response in the DHT-exposed mice might be a consequence of the excessively potent influence of the androgen signaling that overrides the metabolic consequences of GR antagonism. Indeed, GR-responsive genes were previously reported to be subject to regulation by AR, and such a shared regulatory network might underlie why GR antagonism fails to correct disturbances in lipid metabolism in the context of elevated androgen levels (6).

Circadian Rhythm and Glucocorticoid Treatment

In **chapter 3**, we explored the effect of glucocorticoid administration at different times of the day. Our study provides insights into how out-of-phase administration of the synthetic glucocorticoid betamethasone, i.e. administered during the inactive phase when endogenous glucocorticoid levels are low leads to more pronounced disturbances in glucose metabolism compared to in-phase treatment (when glucocorticoid administrations aligns with the natural peak of endogenous glucocorticoids).

We found that out-of-phase betamethasone treatment significantly impairs glucose metabolism. The glucocorticoid-mediated suppression of insulin signaling pathways was reflected by reduced insulin sensitivity and impaired glucose uptake. In humans, circadian misalignment, whether due to disrupted

sleep patterns or shift work, has been associated with insulin resistance and impaired glucose tolerance (41,42), but it is unclear if disturbed glucocorticoid rhythm is involved in these effects. Similarly, circadian misalignment (12-hour behavioral cycle inversion) such as shift work impairs glucose tolerance via separate mechanisms related to insulin secretion and insulin sensitivity in human (43). Our study extends these findings by exploring that both in-phase and out-of-phase synthetic glucocorticoid administration, providing a distinct insight of how exogenous glucocorticoids such as betamethasone disrupt glucose metabolism.

We observed higher plasma insulin levels (i.e. hyperinsulinemia) in response to out-of-phase treatment, reflecting the body's attempt to compensate for the reduced insulin sensitivity by increasing insulin release (which we confirmed by c-peptide measurements). However, the increase in insulin seemed insufficient to maintain normal glucose metabolism, leading to impaired glucose clearance. In addition, out-of-phase treatment increased glucose and triglyceride uptake patterns in some tissues such as gonadal white adipose tissue (gWAT) and liver. This suggests an adaptive response to hyperinsulinemia, but with long-term detrimental effects on glucose handling and insulin sensitivity. (44,45).

It is important to note that the time of measurement greatly influences the measured values for glucose metabolism and insulin levels. This is illustrated by our morning measurements (ZT7) that showed a sharp increase in insulin resistance following out-of-phase betamethasone treatment, while evening measurements (ZT15) revealed different patterns of glucose tolerance and insulin sensitivity. The decision to use different time points for oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) allows for a nuanced understanding of how betamethasone affects glucose metabolism differently at various points in the circadian cycle.

The rhythmic synchronization of glucocorticoid signaling generally allows the body to maintain better glucose homeostasis with relatively higher insulin sensitivity across metabolic tissues, limiting the extent of metabolic disruption (46,47). We found that in-phase (ZT10) betamethasone treatment generally induced less pronounced metabolic disturbances, as compared to out-of-phase administration. Similarly in humans, administrated hydrocortisone caused more potent metabolic effects including elevated glucose and insulin in the evening (when endogenous glucocorticoid levels are low in humans) rather than in the morning (48). In-phase hydrocortisone treatment still caused a reduction in insulin sensitivity, but this effect was milder compared to out-of-phase treatment, altogether in line with our study in mice.

Beyond glucose metabolism, betamethasone treatment also had potent effects on body composition. Our study showed that both in-phase and out-of-phase treatments similarly reduced lean body mass, potentially reflecting glucocorticoid-induced muscle protein breakdown (49). It is noted that hindlimb muscles, especially in male mice, might be less sensitive to the

catabolic action of glucocorticoids than other muscle types (50,51). Glucocorticoid-induced muscle atrophy is not uniform in all muscle types and are influenced by multiple factors such as timing of treatment, sex, and muscle-specific properties (15,52).

In our study, males exhibit greater metabolic disturbances following out-of-phase betamethasone treatment, possibly due to the interaction between testosterone and glucocorticoid. Consistently, deprivation of androgen increases glucocorticoid-induced insulin resistance and fat accumulation in male mice (37), highlighting the intersection of sex hormones and glucocorticoid signaling in metabolic outcomes. Moreover, time of glucocorticoid administration has prominent effects on lipid metabolism and behavioral resultants in rats (53). Although we did not observe significant difference between in-phase and out-of-phase betamethasone treatment on lipid metabolism, which could be partly attributed to the species difference and variation of specific glucocorticoids, our findings are in line with the observation that in-phase delivery of glucocorticoids causes fewer metabolic disturbances, suggesting that timing-based strategy may alleviate certain side effects of chronic glucocorticoid exposure.

Conclusion and Future Directions

In this thesis, we expand knowledge of glucocorticoid signaling in metabolic diseases by pointing out differences between sexes, the function of circadian rhythms, and investigation into the therapeutic potential of modulation of GR. Indeed, all of our studies uniformly showed that exposure to glucocorticoids results in muscle atrophy and metabolic dysfunction, but outcomes were highly divergent between males and females. The stronger transcriptional response in male mice and more severe functional impairments in female mice underlines the necessity for further investigation that govern these sex-specific responses. Androgen signaling was shown to provide some protective effects against glucocorticoid-induced muscle wasting in males, whereas the relatively weaker influence of estrogen on muscle maintenance in females suggests that sex hormones play distinct modulatory roles in the effects of glucocorticoids.

Further studies will be necessary to investigate the molecular pathways underlying sex differences in glucocorticoid response. Exploring how androgen and estrogen receptors interact with the GR in different tissues is an important requirement for developing sex-specific therapies. Moreover, further study is required to delineate non-genomic pathways, epigenetic regulation, and tissue-specific receptor dynamics that mediate these differences, and many of these aspects were not explored or discussed in detail in this thesis.

In **chapter 3**, we investigated the timing of glucocorticoid administration to explored the complexity in glucocorticoid therapy. Glucocorticoids

administered during the active phase of the circadian cycle in mice (morning) resulted in less metabolic disturbance compared to glucocorticoid treatment in the inactive phase of the circadian cycle (evening). This finding points toward a role of circadian biology in the outcome of glucocorticoid therapy, particularly with regard to glucose metabolism. These data suggest that optimization of the timing of glucocorticoid administration might reduce side effects such as insulin resistance and hyperglycemia, which are common in patients undergoing long-term glucocorticoid treatment. Future clinical research requires to translate these preclinical observations into humans for identification of optimum treatment schedules to reduce metabolic adverse effects.

Our study focused on relatively short-term glucocorticoid exposure, and the long-term impact of chronic glucocorticoid use on muscle function, glucose metabolism and general metabolic health are poorly understood. Longitudinal studies which follow glucocorticoid treatment over an extended period of time especially in aging populations are needed, to determine if early metabolic disturbances lead to irreversible changes in humans or the body could adapt over time. This might also elucidate how intermittent versus continuous glucocorticoid therapy affects long-term metabolic outcomes.

Targeting glucocorticoid signaling is also a potential therapeutic strategy for metabolic disorders. In **Chapter 4,** the role of GR antagonism in the PCOS model was discussed. Although GR antagonism had only minor effects on lipid accumulation, it did improve glucose metabolism through early administration in disease development. These findings suggest that timely intervention in hyperandrogenic states may prevent or attenuate many of the metabolic disturbances that occur as a result of excess androgen and glucocorticoid signaling. However, the observation that therapeutic GR antagonism was less effective after metabolic dysfunction had fully developed underlines the early diagnosis and intervention in metabolic diseases is important. It also suggests that other compensatory pathways including androgen signaling may predominate at the late-stage disease, rendering GR antagonism less effective. This now raises questions about combination therapies that target both GR and androgen receptors in disorders such as PCOS in which both pathways are dysregulated.

Personalization of glucocorticoid therapy was one of the important takeaways from this thesis. The dosage of glucocorticoid treatment should consider sex, circadian rhythms, and hormonal status. Biomarkers predicting individual susceptibility to glucocorticoid-induced side effects may be explored in future research. For instance, measuring androgen or estrogen levels and assessing circadian rhythm markers could help clinicians identify the ideal timing of glucocorticoid administration for each patient. Although this thesis investigated glucocorticoid signaling in animal models, it is crucial to translate these findings to human contexts. Future studies should explore whether sex differences and circadian influences on glucocorticoid metabolism affect human physiology or

behavior. Clinical trials incorporating both sex and timing in glucocorticoid treatment regimens will be essential to optimizing therapeutic outcomes across populations.

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