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Molecular regulation of urea cycle function by the liver glucocorticoid receptor

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ABSTRACT

Objective: One of the major side effects of glucocorticoid (GC) treatment is lean tissue wasting, indicating a prominent role in systemic amino acid metabolism. In order to uncover a novel aspect of GCs and their intracellular-receptor, the glucocorticoid receptor (GR), on metabolic control, we conducted amino acid and acylcarnitine profiling in human and mouse models of GC/GR gain- and loss-of-function.

Methods: Blood serum and tissue metabolite levels were determined in Human Addison's disease (AD) patients as well as in mouse models of systemic and liver-specific GR loss-of-function (AAV-miR-GR) with or without dexamethasone (DEX) treatments. Body composition and neuromuscular and metabolic function tests were conducted *in vivo* and *ex vivo*, the latter using precision cut liver slices.

Results: A serum metabolite signature of impaired urea cycle function (i.e. higher [ARG]:[ORN + CIT]) was observed in human (CTRL: 0.45 ± 0.03, AD: 1.29 ± 0.04; $p < 0.001$) and mouse (AAV-miR-NC: 0.97 ± 0.13, AAV-miR-GR: 2.20 ± 0.19; $p < 0.001$) GC/GR loss-of-function, with similar patterns also observed in liver. Serum urea levels were consistently affected by GC/GR gain- (∼+32%) and loss (∼−30%) -of-function. Combined liver-specific GR loss-of-function with DEX treatment revealed a tissue-autonomous role for the GR to coordinate an upregulation of liver urea production rate *in vivo* and *ex vivo*, and prevent hyperammonaemia and associated neuromuscular dysfunction *in vivo*. Liver mRNA expression profiling and GR-cistrome mining identified Arginase I (ARG1) a urea cycle gene targeted by the liver GR.

Conclusions: The liver GR controls systemic and liver urea cycle function by transcriptional regulation of ARG1 expression.

Keywords Amino acid; Protein; Metabolism; Stress

1. INTRODUCTION

Glucocorticoid (GC) treatment remains an effective, and thus oft prescribed, immunosuppressive therapy [1]. However, there are several negative side effects of GC treatment such as lean tissue wasting, osteoporosis, impaired wound healing, increased rates of infection and heightened markers of the metabolic syndrome such as hyperglycaemia and hypertriglyceridaemia [2–4]. Thus, there is a need for either targeted glucocorticoid therapies or identification of additional therapeutic agents/strategies limiting the unwanted side-effects of chronic GC treatment. Nonetheless, there is first a need to gain further insight into the biology of GC side effects in order to better devise such strategies.

While endogenous glucocorticoids signal through both the steroid-hormone receptors, namely the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) [2], a commonly prescribed glucocorticoid (aka Dexamethasone) is a potent GR agonist *in vivo*, with much greater affinity for the GR versus MR [5]. In former studies, we and others have identified tissue-specific functions of the GR in controlling glucose [6–8], lipid [9,10], protein/amino acid [11,12], bile acid [13] and bone [14] metabolism as well as systemic energy homeostasis [13,15–17]. However, whether the liver GR contributes to other aspects of systemic GC action remains enigmatic. To reveal a novel aspect of liver GR function, we conducted amino acid and acylcarnitine profiling studies and uncovered an amino acid profile signature suggestive of altered urea cycle function. Ultimately, we demonstrated a novel role of the liver GR to activate the expression of the key urea cycle enzyme Arginase I, ultimately impacting endogenous amino-N disposal during DEX treatment, thereby affecting hyperammonaemia and associated neuromuscular dysfunction.

2. MATERIALS AND METHODS

2.1. Human experiments

Serum samples were obtained from patients with Addison's disease after acute withdrawal from treatment and from carefully matched

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Received July 15, 2015 • Revision received July 20, 2015 • Accepted July 23, 2015 • Available online xxx

http://dx.doi.org/10.1016/j.molmet.2015.07.006

control subjects, as outlined in detail [18]. In particular, the subset of samples analyzed were taken under fasting or 2 h after ingestion of an high-energy meal. Samples were stored at −80 °C until analysis and freeze-thawing was minimal. Human liver slice studies were conducted as described previously [13].

2.2. Mouse experiments

In all cases, mice were acclimated to the housing facility (12-12h light−dark cycle, 22−24 °C) for at least one week prior to experimentation. Animal experiments were conducted according to local, national, and EU ethical guidelines and approved by local regulatory authorities. To examine the effects of long-term liver GR knockdown, C57 L/J male mice were administered $5 \cdot 10^{11}$ viral genomes of non-specific negative control (NC miRNA) or GR-specific (GR miRNA) miRNA bearing adeno-associated virus as described in detail [13]. To examine the importance of GR dimerisation, 7−10wk old male homozygous glucocorticoid receptor dimerisation deficient ($GR^{dim/dim}$) [19] and wild-type littermate mice were treated daily with 9α-Fluoro-16α-methylprednisolone (Dexamethasone, DEX; 1 mg/kg, D8893 Sigma−Aldrich, DEU) or Vehicle (VEH, 2% ethanol in isotonic saline) for 14d [13]. Furthermore, 8wk old C57 L/J male mice were also chronically treated with DEX as described [13].

To examine the effects of genetic hepatocyte-specific knockout of GR in mice GR floxed/floxed mice [20] were used. In particular, 7−9wk old GR floxed/floxed male mice were administered 1.10^9 ifu of control empty adenovirus (Ad-NC) or an adenovirus to transduce the expression of Cre-recombinase (Ad-CRE). Following 2wk, mice were then administered DEX or VEH as above for 14d. At d7, an intraperitoneal alanine tolerance test (ipATT) was conducted. At d10, the rotarod test was conducted and at d13 grip strength testing and body composition (ECHO-MRI; Echo Medical Systems, USA) were assessed. On d14, mice were sacrificed in the ad libitum fed state and blood and tissues were harvested.

2.3. *In vivo* experiments

To assess the capacity of the liver for urea production *in vivo*, we conducted an alanine tolerance test essentially as performed previously [21]. To this end, mice were fasted overnight and, after a basal blood sample was drawn, intraperitoneally injected with 1 mg/kg alanine solution in 0.9% saline. Additional blood samples were drawn at 1, 2 and 4 h after injection, and blood serum was subsequently analysed for urea concentration. Urea area under the curve was then calculated from 0 mM.

Neuromuscular function was assessed using a grip strength tester (BIO-GS3, BIOSEB, Vitrolles Cedex, France) with the average of two measurements taken. The constant-speed rotarod assay to measure forelimb and hindlimb motor coordination and balance was performed using the rotarod apparatus (Ugo Basile, Biological Research Apparatus, Varese, Italy) as described [22]. After a training period, mice then received several trials at 35 rpm rotation speed with 5-min intervals between individual trials, and the mean latency to fall off the rotarod (for the two trials at each speed level) was recorded and used in subsequent analyses.

2.4. *Ex vivo* liver studies

These experiments were carried out on precision-cut liver slices as previously described [23]. In particular, liver slices were taken and prepared in UW solution (in absence of Insulin and Dexamethasone) and then pre-incubated in Williams media E containing 50 μg/mL gentamycin, 5% dialyzed FBS, 11 mM D-glucose, 0.3 mM pyruvate, 0.1 μM methyl-linoleate, and an amino acid mixture resembling the hepatic portal vein concentrations [24]. After 1 h, media was switched to that containing either vehicle (0.4% DMSO + 0.01% EtOH), dexamethasone (1 μM in 0.4% DMSO + 0.01% EtOH), RU486 (10 μM in 0.01% EtOH + 0.4% DMSO) and dexamethasone + RU486 (1 & 10 μM respectively in 0.4% DMSO + 0.01% EtOH) and incubated for 18 h after which media and liver samples were taken and snap frozen in LN_2. To examine the role of Arginase I (ARG1) activity on DEX modulated urea cycle function, liver slices were prepared and treated with DEX as above with and without two structurally dissimilar ARG1 inhibitors, Nω-Hydroxy-nor-L-arginine (L-NOHA; ALX-270-467, Enzo Life Sciences, DEU) and S-(2-Boronoethyl)-L-cysteine (BOC; ALX-270-345, Enzo Life Sciences, DEU), at concentrations (i.e. 100 μM) known to affect cellular urea cycle function [25]. Importantly, for all studies, media without liver sample was also incubated to serve as a metabolite assay control. Metabolite balance rates were calculated as media concentrations from liver slice incubations minus those from media without slice incubations, divided by the time of incubation (i.e. (slice media (M) − control media (M))/time (h)). Importantly, liver slice weights (4.2 ± 0.1 mg) did not differ between any of the conditions studied.

2.5. Recombinant viruses

Adenoviruses were produced according to the Life Technologies manual and titered via the serial dilution plaque-formation unit titration assay (Virapower Adenoviral Expression System). Adenoviruses were purified by the caesium chloride method and dialyzed against phosphate-buffered-saline containing 10% glycerol prior to animal injection.

For long-term inactivation of GR, recombinant adeno-associated viruses (AAV) encoding GR-specific miRNA were established and produced as outlined in detail by Rose et al. [13]. Importantly, using an hepatocyte-specific promoter [26] this method allows for hepatocyte restricted transduction/expression [13].

2.6. Metabolite measurements

Blood glucose levels were determined using an automatic glucose monitor (One Touch, Lifescan). In addition, commercially available kits were used to measure serum urea (Z5030016, Biochain, USA) and ammonia (K370-100, Biovision, USA) according to manufacturer's instructions. All samples were loaded in order to fit within the assay range of the reagents supplied. Amino acids and acylcarnitines were determined in serum by electrospray ionization tandem mass spectrometry (ESI-MS/MS) according to a modified method as previously described [27], using a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source and a Micromass MassLynx data system. For liver determinations, frozen pulverised liver was carefully weighed and then extracted in 10 volumes of ice cold PBS. Values obtained were then calculated to give μmol/g wet liver tissue weight.

2.7. RNA extraction and analysis

Total RNA was extracted from homogenized mouse liver or cell lysates using Qiazol reagent (Qiagen, Hilden, DEU). cDNA was prepared by reverse transcription using the M-MuLV enzyme and Oligo dT primer (Fermentas, St. Leon-Rot, DEU). cDNAs were amplified using assay-on-demand kits and an ABIPRISM 7700 Sequence detector (Applied Biosystems, Darmstadt, DEU). RNA expression data was normalized to levels of TATA-box binding protein (TBP) RNA. Particular product numbers for probe sets can be provided upon request.

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2.8. Tissue protein extraction and analysis

For simple expression analyses from homogenates, protein was extracted from ~50 mg frozen liver powder in tissue lysis buffer [28]. To determine protein expression, total protein concentration was analyzed by BCA assay (23227, Fischer Scientific, DEU) and equal amounts of protein were loaded onto an SDS-polyacrylamide gel, subjected to electrophoresis and blotted onto nitrocellulose membrane. Western blot assays were performed using standard techniques with antibodies specific for the glucocorticoid receptor (GR; sc-1004, Santa Cruz Biotech., Heidelberg, DEU), Arginase I (ARG1; sc-20150, Santa Cruz Biotech., Heidelberg, DEU) or valosin-containing protein (Vcp; ab11433, Abcam, Cambridge, UK). For expression analyses, all western blots signals were within a linear range, with respect to protein loaded and antibodies used, as determined by preliminary experiments, and detected bands ran at the expected relative mobility (data not shown).

2.9. Liver GR DNA binding *in vivo*

Publically available sequencing data [29] were analysed *in silico* in order to ascertain DEX-dependent GR-DNA binding regions. Accessible NCBI Gene Expression Omnibus GSE files for GR & CEBPB Chip-Seqs and DNAse Hypersensitivity were downloaded and uploaded to Ensembl Mouse Genome Browser NCBI m37. The browser was then configured to display Arginase 1 gene and promoter regions. In addition, accessible NCBI Gene Expression Omnibus GSE files for GR Chip-Seq were downloaded and mapped to the mouse mm10 genome by using Bowtie [30]. Subsequently, the mapped reads were analysed with MACS2 software [31] to identify GR chips peaks. The input files were used as control. The wiggle files of GR chip and input control were uploaded to Integrative Genomics Viewer (IGV 2.3; [32]) to display Arginase 1 gene and promoter regions.

2.10. Statistical analysis

Statistical analyses were performed using t-tests, or 2-way analysis of variance (ANOVA) with or without repeated measures, where appropriate, with Holm–Sidak-adjusted post-tests. All analyses were carried out with SigmaPlot v.12 software (Systat Software GmbH, DEU).

3. RESULTS

Amino acid profiling reveals altered urea cycle function by the HPA-liver GR axis in human and mouse. To examine whether dysfunctional HPA axis and thus whole body glucocorticoid action affects novel aspects of metabolic function, we conducted amino acid/acylcarnitine profiling in Addison's patients versus aged-matched controls. In the fasted state, analysis of the serum from Addison's patients revealed higher amounts of tyrosine (TYR) and arginine (ARG), lower amounts of ornithine (ORN) and citrulline (CIT), and no change in any other amino acid (Figure 1A and Figure. S1A) or acylcarnitine species (Figure S1B). In particular, there was a heightened ratio of serum [ARG]:[ORN + CIT] in Addison's patients (1.29 ± 0.04) versus controls (0.45 ± 0.03; $p < 0.001$). Given that ARG, ORN and CIT are key metabolites within the urea cycle [33], we then examined serum urea, and we observed lower serum urea in Addison's patients. Importantly, this pattern was observed whether we examined serum taken from the postabsorptive or postprandial state (data not shown). In contrast to Addison's patients, whose condition is characterized by low circulating glucocorticoid levels [18], chronic treatment with the glucocorticoid-like drug dexamethasone (DEX) resulted in higher serum urea levels in mice (Figure 2A). As dexamethasone is a selective glucocorticoid receptor (GR) agonist [5], we tested whether there was

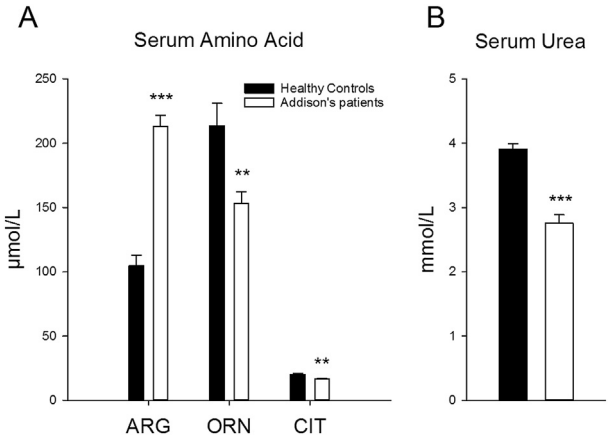

Figure 1: Serum amino acid profiling reveals a pattern of disturbed urea cycle function in Addison's patients. Amino acid (A) arginine (ARG), ornithine (ORN) and citrulline (CIT) as well as urea (B) levels were measured from serum samples collected from individuals with Addison's disease following acute treatment withdrawal and from matched healthy control individuals. Data are mean ± SEM; n = 9 per group. Difference between groups: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

an interaction between DEX treatment and GR function by examining the response to DEX in whole-body GR loss-of-function transgenic mice, which express a mutant GR incapable of dimerization function [19], and thus transcriptional effectiveness of the GR [19], particularly through canonical palindromic GR DNA binding motifs [35]. In particular, while DEX resulted in higher serum urea, this effect was absent in GR^{dim} mice (Figure 2B), meaning that the effect of DEX to increase urea cycle rate is most likely conferred by systemic GR action. Given that the complete urea cycle *in vivo* mainly occurs in the liver parenchyma [33], we then tested whether urea cycle function is also affected in a mouse model of liver hepatocyte GR loss-of-function (AAV-miR-GR) [13]. Indeed, serum urea (Figure 2C) and the serum levels of the urea cycle metabolites ARG (↑), ORN (↓) and CIT (↓) were affected (Figure 2D) with a heightened ratio of serum [ARG]:[ORN + CIT] in AAV-miR-GR (2.20 ± 0.19) versus AAV-miR-NC (0.97 ± 0.13; $p < 0.001$). Importantly, a similar pattern was observed in the liver (Figure 2E). Furthermore, hepatocyte-specific GR loss-of-function resulted in higher amounts of TYR in serum and liver with several other amino acid (Figure S2A–B) and acylcarnitine species (Figure S2C–D), such as MET, GLN/GLU, and GLY, also being affected. Altogether, these data indicate that the liver GR modulates urea cycle function *in vivo*.

Hepatic glucocorticoid resistance impairs the upregulation of urea cycle resulting in hyperammonaemia and neuromuscular dysfunction upon dexamethasone treatment. Conceivably, changes in urea cycle function *in vivo* upon GR gain- and loss-of-function could result from tissue autonomous or tissue non-autonomous mechanisms. To investigate this further, we performed studies using mouse liver slices *ex vivo*, which, unlike primary hepatocytes, maintain glucocorticoid sensitivity and more closely resemble fully differentiated liver *in vivo* [23]. We treated mouse liver slices with DEX and the GR-antagonist RU486, and combinations thereof, and could demonstrate that while DEX promoted liver urea production rate, as well as net ORN output and net ARG and TYR uptake, RU486 completely inhibited this effect (Figure 3A–C; Figure S3A), demonstrating that the liver GR action *per se* is sufficient to confer effects of DEX on urea cycle function. Given this result, we wanted to test whether the liver GR could confer systemic effects of DEX treatment *in vivo*. Indeed, liver GR loss-of-function (Figure S3B) had expected effects on blood glucose

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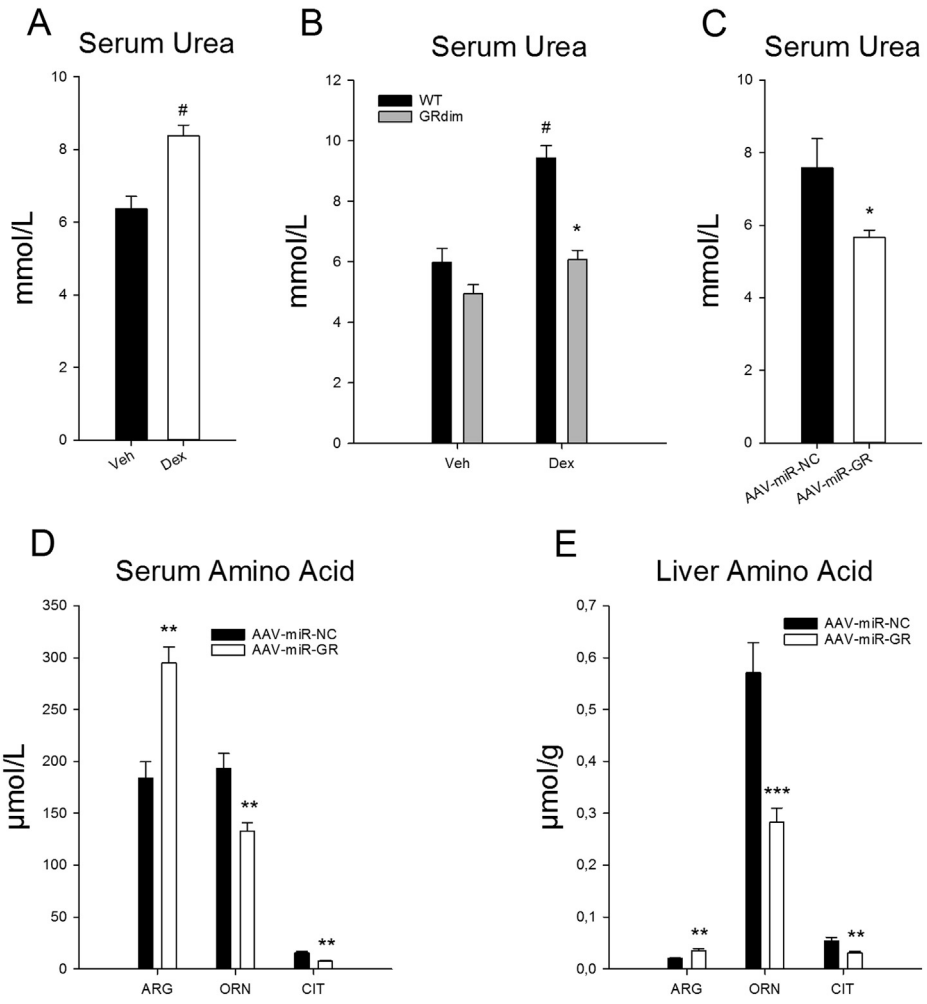

Figure 2: Mouse models of GR loss- and gain of function reveal a role from the liver glucocorticoid receptor in controlling urea cycle function. A: Serum urea levels from mice chronically treated with Dexamethasone (Dex) or corresponding Vehicle (Veh); n = 4/group. B: Serum urea levels from whole-body $GR^{dim/dim}$ or wildtype (WT) littermate mice chronically treated with Dex or corresponding Veh; n = 4–5/group. C: Serum urea (C), amino acid (D) as well as liver amino acid (E) levels in mice pre-treated with adeno-associated virus to express a negative control miR (AAV-miR-NC) or GR-specific miRNA (AAV-miR-GR); n = 9/group. Data are mean ± SEM. Effect of genotype/knockdown: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Effect of Dex: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

(Figure S3C), but also dramatically reduced DEX-induced *in vivo* urea production rate, as judged by an alanine tolerance test (Figure 3D–E). Importantly, serum (Figure S3F) and liver (Figure S3G) amino acid profiling revealed that while DEX promoted lower ARG and higher ORN and CIT levels, these effects were blunted by liver-specific GR knockdown, highlighting a key role for the liver in mediating these changes. We then examined the potential functional consequences of GR loss-of-function in this study, and could demonstrate that the liver GR does not affect the reduction of body mass that typically occurs with DEX treatment *in vivo* (Figure 4A–B). In particular, ECHO-MRI revealed that DEX reduced lean mass (Figure 4C) but not fat mass (Figure S4A), with lower skeletal muscle (Figure S4B) and liver (Figure S4C) mass upon DEX treatment. We hypothesised that if the urea cycle regulation were dysfunctional, this would lead to hyper-ammonaemia upon DEX treatment, and, indeed, this was the case (Figure 4D). Given that hyperammonaemia typically results in a manifestation of neuromuscular dysfunction [34], we tested this possibility and could demonstrate that both forelimb grip strength (Figure 4E) as well as balance control (Figure 4F) were negatively affected by DEX treatment in liver GR knockout mice. Taken together, these data demonstrate that the liver GR coordinated effective amino acid disposal via tissue-autonomous upregulation of urea cycle function upon DEX-induced lean tissue wasting.

Liver Arginase I is a GR-target gene. Conceivably, the GR can confer function by either genomic or non-genomic mechanisms. However, since we could observe GR dimerization dependent effects of DEX treatment (Figure 2B), we hypothesised that GR-conferred function occurs most likely via transcriptional events, as GR dimerization-deficient mutants impair transcriptional effectiveness of the GR [19], particularly through canonical palindromic GR DNA binding motifs [35]. Hence, as there are only several key gene transcripts within the liver urea cycle [33], we performed a focused transcript analysis. In particular, the transcripts Cps1, Otc, Slc25a15, Ass1 and Asl were not affected by liver/hepatocyte GR knockdown, but liver *Arg1* mRNA expression was ~50% lower (Figure 5A). Importantly, we could confirm this result at the level of ARG1 protein expression (Figure 5B). Furthermore, although DEX induced liver *Arg1* expression *in vivo* (Figure 5C) and *ex vivo* (Figure 5D–E), these effects were blocked by GR loss-of-function. A known [36] GR target gene Tyrosine amino-transferase (*Tat*) was also assessed, which confirmed the

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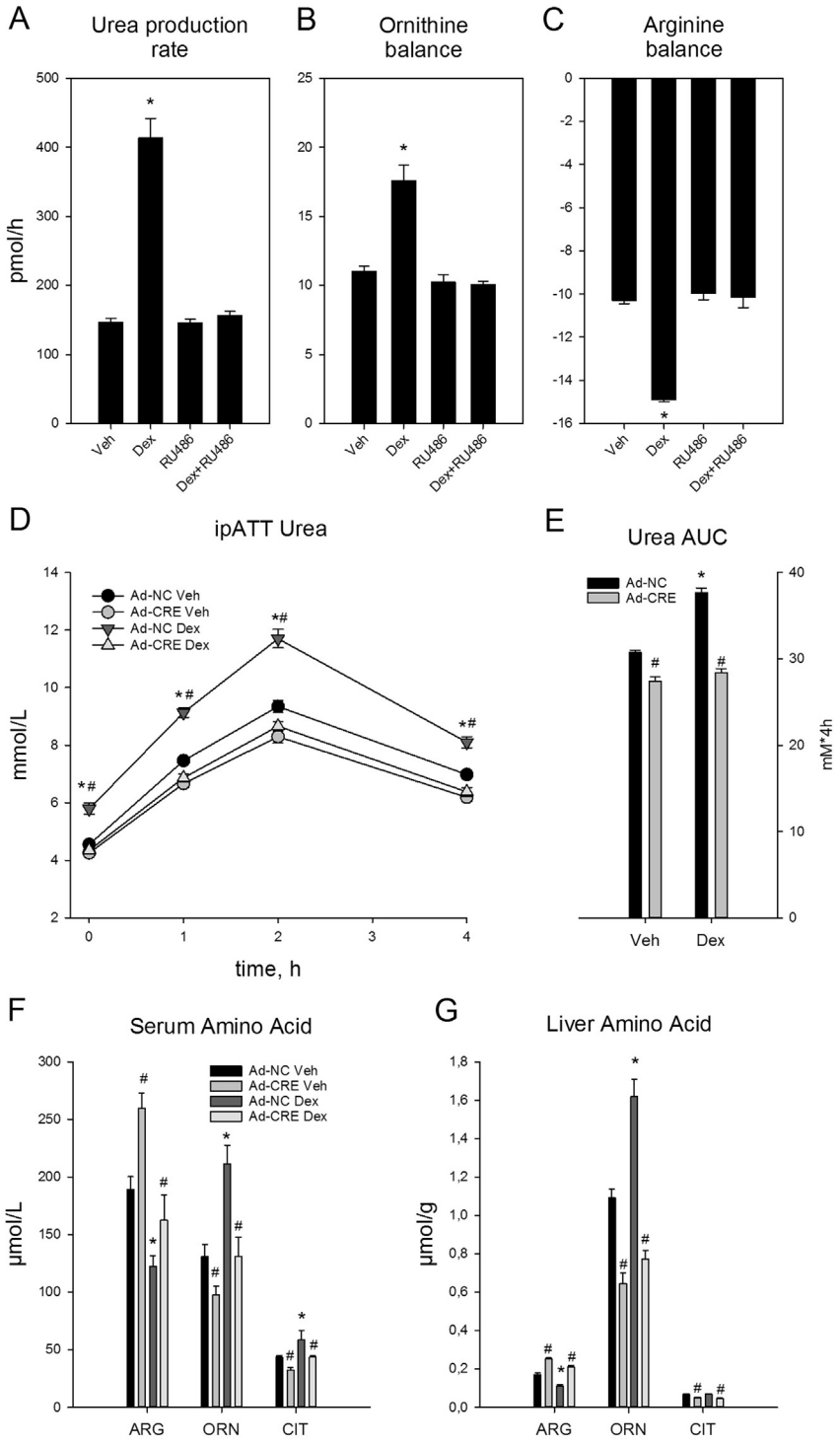

Figure 3: The liver glucocorticoid receptor coordinates the enhanced urea cycle function upon dexamethasone treatment. Urea production rate (A) as well as ornithine (B) and arginine (C) balance in mouse liver slices treated with dexamethasone, RU486 and corresponding vehicle treatments *ex vivo* (N = 4/group). Serum urea levels (A) and urea area under the curve (AUC; B) in response to an intraperitoneal alanine tolerance test (ipATT) in GR-floxed mice pretreated with adenoviral constructs expressing (Ad-CRE) or not (Ad-NC) Cre-recombinase with (Dex) or without (Veh) chronic dexamethasone treatment (N = 6–7/group). Serum (F) and liver (G) amino acid levels in the same mice as in D. ARG: arginine, ORN: ornithine, CIT: citrulline. Data are mean ± SEM. Effect of Dex: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Effect of Ad-CRE: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

effectiveness of the *ex vivo* model used (Figure S5A). The upregulation of ARG1 by the liver GR is likely of functional importance, as inhibition of ARG1 by two distinct inhibitors largely blunted the upregulation of liver urea production rate *ex vivo* (Figure 5F). Lastly, to understand how the GR might regulate the *Arg1* gene transcription, we examined pre-existing liver GR ChIP-seq data [29]. Importantly, we could demonstrate that DEX treatment *in vivo* upregulated GR binding to a region within intron 1 of the *Arg1* gene (Figure 5G), which contains a

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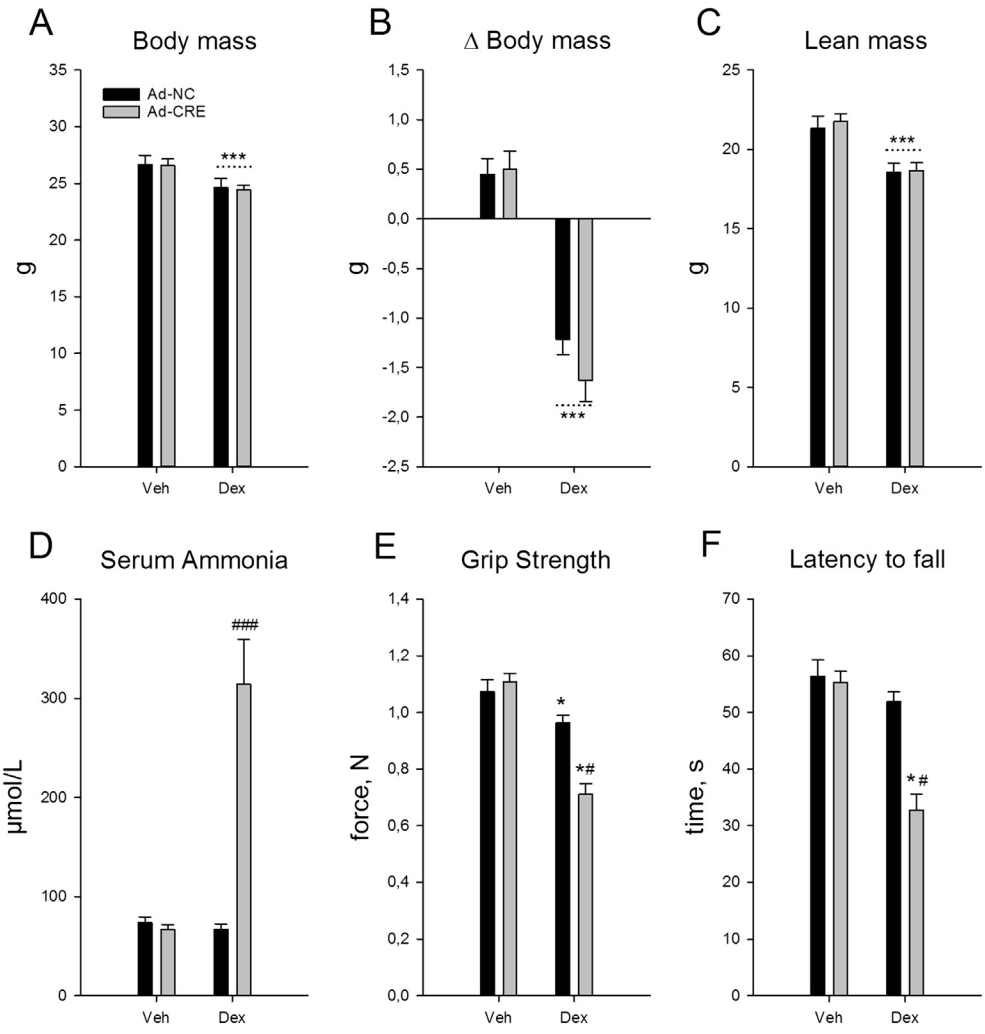

Figure 4: Hyperammonaemia and dysfunctional neuromuscular function coincide with improper regulation of liver urea cycle function with liver GR loss in dexamethasone treatment. End-point (A) and delta (B) body mass and lean mass (G) in GR-floxed mice pre-treated with adenoviral constructs expressing (Ad-CRE) or not (Ad-NC) Cre-recombinase with (Dex) or without (Veh) chronic dexamethasone treatment (N = 6–7/group). Furthermore, serum ammonia (D) was measured and tests of neuromuscular function such as forelimb grip strength (E) and latency to fall during a rotarod test (F) were conducted. Data are mean ± SEM. Effect of Dex: *p < 0.05, **p < 0.01, ***p < 0.001. Effect of Ad-CRE: #p < 0.05, ##p < 0.01, ###p < 0.001.

consensus glucocorticoid regulatory element (data not shown) corresponding to DNAase hypersensitivity sites (DHS) and CCAAT/enhancer-binding protein β (CEBPβ) binding sites (Figure S5B). Of note, even though there were DHSs and CEBPβ binding sites farther upstream (Figure S5B), there were no consistent GR binding sites regulated by DEX in these regions (not shown).

4. DISCUSSION

In addition to our previously described effects on glucose [8], lipid [9,10], and bile acid [13] metabolism, here we demonstrate that the liver glucocorticoid receptor (GR) coordinates proper urea cycle function in mouse and human. In particular, the liver GR upregulates the expression of Arginase I (ARG1) during dexamethasone (DEX) treatment, aiding in the disposal of excess amino acid N yielded from lean tissue wasting and preventing accumulation of ammonia and associated pathophysiological side effects.

Although the increase in systemic urea cycle function by GR-agonism by DEX has been demonstrated previously in rodents [37,38] and humans [39], and although systemic pharmacological GR blockade blunts trauma-enhanced urea cycle capacity [40], the precise site(s) of control had not been identified. Moreover, it is feasible that the simple increase in hepatic amino acid delivery driven by amino acid release during GC-induced lean tissue wasting, which is mediated by the muscle GR [11,12], could potentially promote ureagenesis by substrate-driven flux [33]; *i.e.* increased liver amino-N supply. However, others have surmised that there is a specific control site in the liver [41], and in accordance with studies of perfused rodent liver [42], we demonstrated a tissue-specific role of the GR by both *ex vivo* liver studies as well as liver/hepatocyte specific GR loss-of-function studies *in vivo*. Furthermore, while others have hypothesised that the DEX driven activation of liver urea cycle function could control the effects on lean tissue wasting [38], we showed that these phenomena are actually disconnected. Of note, we also demonstrated that the loss of GR within the adult animal mediated this effect, thereby negating the argument that the effects that we observe could relate to altered liver-development by the GR, which could occur in conventional knockout mice or knockout mice driven by a promoter that drives expression during liver development.

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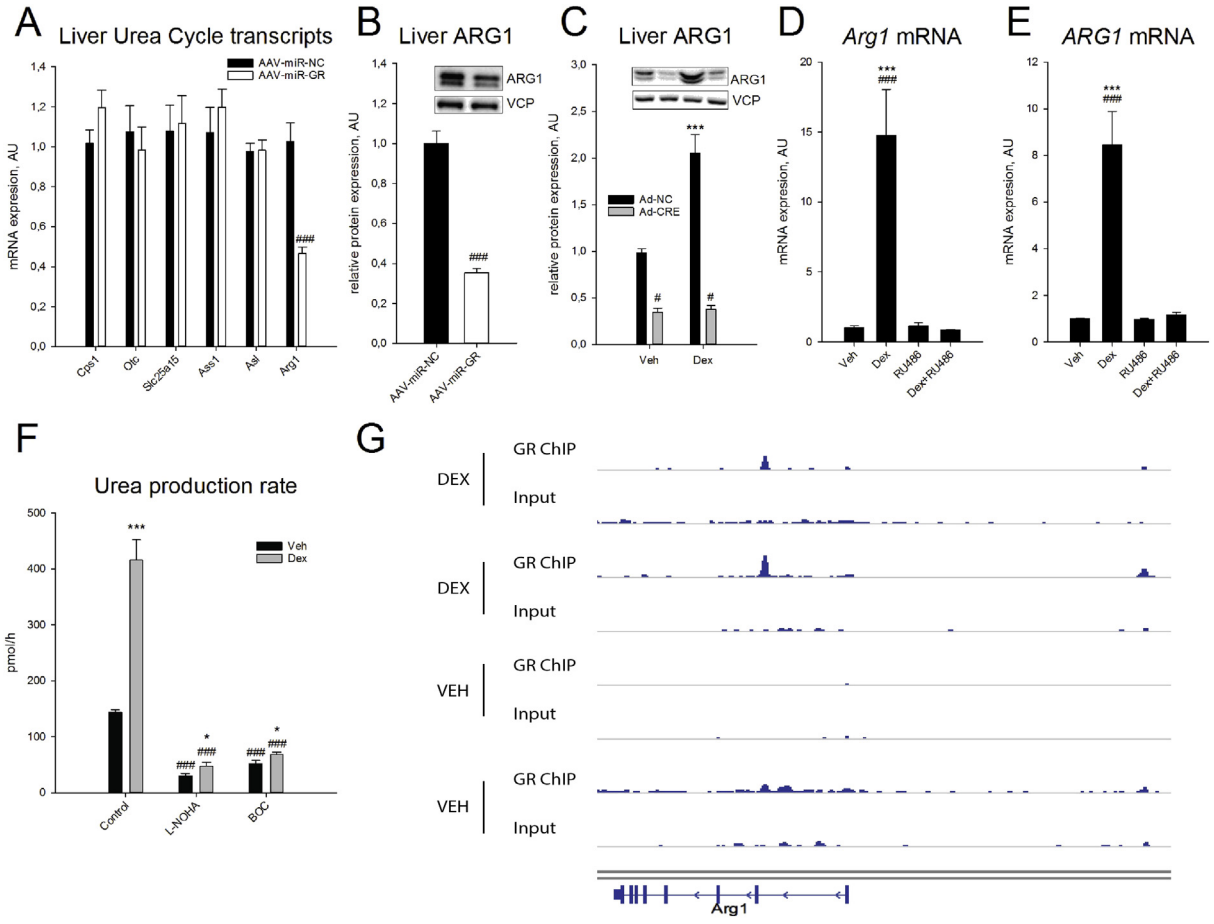

Figure 5: Arginase I is a liver glucocorticoid receptor target gene coordinating proper regulation of urea cycle function upon glucocorticoid exposure. The liver mRNA expression (A) levels of carbamoyl-phosphate synthase 1 (Cps1), ornithine transcarbamylase (Otc), mitochondrial ornithine transporter (Slc25a15), arginosuccinate synthase 1 (Ass1), arginosuccinate lyase (Asl), and arginase 1 (Arg1) in mice pre-treated with adeno-associated virus to express a negative control miR (AAV-miR-NC) or GR-specific miRNA (AAV-miR-GR); n = 9/group. B: Liver arginase 1 protein (ARG1) expression in the same mice as in A. C: Liver ARG1 expression in the same mice as in Figures 3D–G & 4 (N = 6–9/group). D: Liver Arg1 mRNA in mouse liver slices treated with dexamethasone, RU486 and corresponding vehicle treatments *ex vivo* (N = 4/group). E: Liver *ARG1* mRNA in human liver slices treated as in D. Urea production rate of mouse liver slices treated with dexamethasone, with and without ARG1 inhibitors Nω-Hydroxy-nor-L-arginine (L-NOHA; 100 μM) and S-(2-Boronoethyl)-L-cysteine (BOC; 100 μM) *ex vivo* (N = 4/group; F). Glucocorticoid receptor (GR) ChIP-seq peaks around the Arg1 gene of liver of mice treated with (DEX) and without (VEH). Data are mean ± SEM. Effect of Dex: *p < 0.05, **p < 0.01, ***p < 0.001. Effect of knockdown/inhibitor: #p < 0.05, ##p < 0.01, ###p < 0.001.

Here we show that the main mechanism of action likely relates to the upregulation of a key transcript encoding a protein within the hepatic urea cycle, namely Arginase I. Indeed, the metabolic signature in the serum and liver that we observe with GR loss-of-function, i.e. higher arginine and lower ornithine/urea, are direct substrates and products of the ARG1 reaction [33], fitting that this could be a key rate limiting event *in vivo*. In accordance, ARG1 inhibition largely blunted the DEX-induced upregulation of urea production rate by liver *ex vivo*. This is important, as it is thought that CPS1 and ASS1 urea cycle reactions are rate limiting under normal conditions [33]. Nevertheless, it could be that under the particular condition of activation of the stress-response, and hence heightened HPA axis activity, the rise in GC-GR action and consequently whole body protein turnover and AA flux could shift the site of control to another step, in this case ARG1. In any case, that we observe increased serum arginine as well as accumulation of serum ammonia and consequent neuromuscular dysfunction, with the lack of upregulation of liver ARG1 by GR loss, is in accordance with other studies of ARG1 knockout mice [43–45].

Regarding the molecular mechanism by which DEX/GR could upregulate liver urea cycle function, others have demonstrated that several of the urea cycle enzymes are indeed upregulated by GR-agonism *in vivo* [38] and *in vitro* [46]. Regarding ARG1, earlier studies using hepatoma cells have demonstrated a requirement for ongoing protein synthesis for the upregulation of *Arg1* mRNA and have proposed that the there is a two-step mechanism. The first step requires the synthesis of the transcription factor CCAAT/enhancer-binding protein β (CEBPβ), which then binds to an enhancer region downstream of the promoter between exon 7 and 8 [47]. Indeed, CEBPβ is required for DEX-induction of mouse hepatocytes *in vitro*, but not *in vivo*, suggesting a rather complicated mechanism [48]. Regardless, we could demonstrate GR-dependent DEX-induction of *Arg1* mRNA in mouse and human liver-slices *ex vivo*, which is a better model than primary hepatocytes as liver slices retain better glucocorticoid sensitivity (data not shown). More recent CHIP-on-ChIP [49] and ChIP-seq [29] studies have demonstrated direct binding of the GR to a region within the *Arg1* gene in the liver of mice. In particular, these studies have failed to verify the aforementioned CEBPβ region and, indeed, have identified a region within intron 1, which is bound by CEBPβ and displays enhanced GR-binding upon DEX treatment *in vivo* [29], in accordance with current models of transcriptional regulation by the GR [50]. Taken

together, these data suggest that the activated GR directly binds the *Arg1* gene, which perhaps together with CEBPβ [29] and steroid receptor coactivator 1 (SRC1) [51] is sufficient to enhance transcription rate, but do not rule out the possibility that other factors could be involved *in vivo*. Indeed, glucagon and dexamethasone have synergistic actions on *Arg1* transcription [46], and glucocorticoids can increase glucagon levels either via direct [52] or indirect [53] mechanisms.

In addition to effects on the urea cycle metabolites, we consistently observed that the amino acid tyrosine and liver tyrosine aminotransferase (TAT) mRNA expression were affected by DEX and GR loss-of-function *in vivo* and *ex vivo*. Although TAT was already identified as a liver GR target gene [36], perhaps together with SRC1 [51] as well as other as-yet unidentified elements, we now demonstrate that transcriptional control through the liver GR modulates *in vivo* metabolic function of liver TAT, of which likely has physiological consequences *in vivo* [51].

Aside from chronic GC treatment, our results could extend to other conditions of chronic HPA-axis activation such as trauma/surgery, cachexia, type I diabetes, and perhaps obesity-driven type II diabetes [2,3]. Given that major mechanism contributing to diabetes is aberrant hepatic glucose production [54] and amino acids are potential gluconeogenic substrates [55], an enhanced amino acid turnover and urea cycle function may also contribute to impaired glucose control driven by high levels of GC. Indeed, here we observed a correlation between these two processes, and thus this a clear direction for further investigations. In summary, the liver GR controls urea cycle function, at least in part via transcriptional activation of the *Arg1* gene, and thus contributes to amino acid homeostasis under conditions of glucocorticoid excess.

ACKNOWLEDGEMENTS

SC, JS, AZ, OS, AM & AJR performed experiments and analysed/prepared samples. KVS & JGO conducted amino acid and acylcarnitine profiling of samples and edited the manuscript. JK & AP conducted the Addison's patient experiments and edited the manuscript. XW and RdG conducted *in silico* analyses from publically available datasets. SH provided infrastructure and funds and edited the manuscript. AJR designed and directed the research, analysed the data and wrote the manuscript.

We acknowledge Mrs. Katharina Sowodniok, Dr. Frits Mattijssen and Dr. Anja Krones-Herzig for experimental support. Dr. Ilya Vinnikov is gratefully acknowledged for invaluable advice on rotarod testing protocols and for helpful suggestions on the manuscript.

CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.07.006.

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