REV-ERBα influences the stability and nuclear localization of the glucocorticoid receptor

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ABSTRACT
REV-ERBα (encoded by Nr1d1) is a nuclear receptor that is part of the circadian clock mechanism and regulates metabolism and inflammatory processes. The glucocorticoid receptor (GR, encoded by Nr3c1) influences similar processes, but is not part of the circadian clock, although glucocorticoid signaling affects resetting of the circadian clock in peripheral tissues. Because of their similar impact on physiological processes, we studied the interplay between these two nuclear receptors. We found that REV-ERBα binds to the C-terminal portion and GR to the N-terminal portion of HSP90α and HSP90β, a chaperone responsible for the activation of proteins to ensure survival of a cell. The presence of REV-ERBα influences the stability and nuclear localization of GR by an unknown mechanism, thereby affecting expression of GR target genes, such as IκBα (Nfkbia) and alcohol dehydrogenase 1 (Adh1). Our findings highlight an important interplay between two nuclear receptors that influence the transcriptional potential of each other. This indicates that the transcriptional landscape is strongly dependent on dynamic processes at the protein level.

KEY WORDS: Nuclear receptor, Circadian clock, Glucocorticoid receptor, REV-ERBα, HSP90

INTRODUCTION
In mammals, the circadian clock system regulates many physiological, biochemical and behavioral processes with the suprachiasmatic nuclei (SCN) as the main coordinating entity to synchronize all cellular clocks in the body (Panda et al., 2002; Reppert and Weaver, 2002). At the cellular level, the circadian clock mechanism is controlled by genetically determined networks of transcription–translation feedback loops involving clock genes, including period (Per, for which there are three genes Per1–Per3), cryptochrome 1 and 2 (Cry1 and Cry2), Bmal1 (also known as Arntl) and Clock (Reppert and Weaver, 2001). The transcription factors CLOCK and BMAL1 heterodimerize and activate the expression of Per and Cry genes by binding to E-box elements in their promoters. CRY and PER proteins form oligomers that are transported from the cytoplasm to the nucleus, where they repress their own transcription by inhibiting BMAL1–CLOCK activity. BMAL1–CLOCK also induces the expression of the nuclear receptor REV-ERBα (also known as NR1D1), which represses the transcription of Bmal1 through direct binding to a REV-ERBα response element (RORE) within the Bmal1 promoter (Buhr and Takahashi, 2013). Rev-erba (Nr1d1) is expressed in a variety of tissue types, including brown fat, skeletal muscle and liver (Woldt et al., 2013; Everett and Lazar, 2014). In addition to its action in the circadian clock mechanism, REV-ERBα has been implicated in adipogenesis (Kumar et al., 2010; Laitinen et al., 2015), muscle differentiation (Downes et al., 1995), liver metabolism (Bugge et al., 2012; Cho et al., 2012) and neurogenesis (Schnell et al., 2014).

The glucocorticoid receptor (GR, encoded by Nr3c1) belongs to the superfamily of steroid, thyroid and retinoid acid receptor proteins that function as ligand-dependent transcription factors (Evans, 1988). A common feature of GR is an obligate interaction with heat-shock protein 90 (HSP90α and HSP90β, hereafter HSP90) before hormone-dependent activation (Kirschke et al., 2014) and this interaction is also known to be important in regulating GR stability (Siriani et al., 2005; Sultana et al., 2013). Upon binding its cognate ligand (glucocorticoids), GR undergoes conformational changes, dissociates from HSP90 binding, and subsequently translocates to the nucleus and binds to conserved palindromic DNA sequences, the glucocorticoid responsive element (GRE) (Berg, 1989). Depending on the target gene, hormone-activated GR can stimulate or inhibit gene transcription and direct binding of activated GR on GRE is the main mechanism of regulation associated with glucocorticoid-mediated transactivation (De Bosscher et al., 2003). For example, GR exerts anti-inflammatory action in part by antagonizing nuclear factor κB (NF-κB), which is known to activate genes coding for pro-inflammatory cytokines such as tumor necrosis factor (TNF) (De Bosscher et al., 2000) through the upregulation of the expression of the NF-κB inhibitor IκBα proteins (Scheinman et al., 1995; Almawi and Meledem, 2002). Recent studies have also indicated that GR can bind as a monomer or homodimer to target genes, depending on absence or presence of glucocorticoids (Lim et al., 2015).

A role of REV-ERBα in inflammation has been reported (Gibbs et al., 2012, 2014), and REV-ERBα appears to regulate NF-κB-responsive genes in human vascular smooth muscle cells (Migita et al., 2004). Overexpression of REV-ERBα upregulates inflammatory markers, like IL-6 and COX-2, as well as nuclear translocation of NF-κB (Migita et al., 2004), suggesting that REV-ERBα might play an important role in the regulation of NF-κB responsive genes. Among NF-κB controlled genes, TNF plays a crucial role in alcohol liver diseases (Beier and McClain, 2010; Gao et al., 2011). Ethanol increases NF-κB activation through reactive oxygen species (ROS)-dependent pathways (Wang et al., 2015) and production of TNF in hepatocytes is controlled by the transcriptional activation of NF-κB through the degradation of IκBα (Fan et al., 2004).

Given that REV-ERBα might be involved in regulation of NF-κB and that GR exerts anti-inflammatory functions through the upregulation of the expression of IκBα, the correlation between
REV-ERBα and GR is of particular interest. Therefore, we studied the relationship between REV-ERBα and GR. Here, we show that REV-ERBα and GR bind to HSP90 at different sites, affecting their stability and subcellular localization by unknown mechanisms. As a consequence the regulation of target genes is affected.

RESULTS

REV-ERBα influences the GR at the protein but not at the transcript level

In order to test the possibility of whether GR and REV-ERBα influence each other, we tested the expression of GR in Rev-erbα−/− animals and compared it with the expression in wild-type (WT) animals using liver extracts. Western blotting revealed that GR protein was expressed in an almost constant manner over 1 day in the liver and that this expression was strongly increased in Rev-erbα−/− animals (Fig. 1A). Quantification of several experiments indicated that this increase was statistically significant at the time points zeitgeber time (ZT) 0, 12 and 18, where ZT0 is lights on and ZT12 is lights off (Fig. 1B). This suggests that Rev-erbα affects GR protein accumulation.

As a next step, we tested the possibility that REV-ERBα regulates the expression of GR (Nr3c1) at the transcriptional level. Therefore, we extracted mRNA from livers of wild-type and Rev-erbα−/− mice. Quantitative real-time PCR (qRT-PCR) revealed that the level of Nr3c1 mRNA was similar in wild-type and Rev-erbα−/− livers (Fig. 1C), whereas Rev-erbα mRNA was absent in the Rev-erbα−/− animals (Fig. 1D). Given that the murine Nr3c1 promoter contains REV-ERBα-binding sites (RORES, Fig. 1E), we tested whether this promoter cloned in front of a luciferase reporter gene (Nr3c1::luc) was repressed by REV-ERBα in a transfection experiment using NIH3T3 cells. We observed that increasing concentrations of Rev-erbα expression vector did not lead to a reduction of luciferase activity when using the Nr3c1::luc reporter in contrast to the positive control Bmal1::luc reporter (Fig. 1F). This result indicates that the Nr3c1 promoter is not directly regulated by the repressor activity of REV-ERBα, which is consistent with the unaltered levels of Nr3c1 mRNA in Rev-erbα−/− mice (Fig. 1C). Therefore, the increase in GR protein in the liver of Rev-erbα−/− animals probably occurs post-transcriptionally and/or post-translationally.

GR and REV-ERBα both bind to HSP90 affecting GR stability

Lack of the Rev-erbα gene leads to increased GR protein in the liver (Fig. 1A). If REV-ERBα affected GR levels post-transcriptionally, overexpression of Rev-erbα would lead to a reduction of GR protein. In order to test this hypothesis, we overexpressed differing amounts of REV-ERBα in NIH 3T3 cells. We observed that increasing amounts of REV-ERBα protein lead to a reduction of GR protein in these cells as revealed by western blotting (Fig. 2A). One explanation for this might be that GR is less stable in presence of excess REV-ERBα. Because HSP90 interacts with GR and thereby stabilizes it (Sirianni et al., 2005; Sultana et al., 2013), we hypothesized that REV-ERBα might also bind to HSP90 and modulate GR stability. Therefore, we immunoprecipitated HSP90 in presence of increasing amounts of REV-ERBα, and tested the amounts of REV-ERBα and GR, respectively, that were co-precipitated with HSP90 by western blotting (Fig. 2B). Interestingly, increasing amounts of REV-ERBα reduced the amount of GR that was co-precipitated with HSP90 and increased the amount of REV-ERBα that was co-precipitated with HSP90 in a dose-dependent manner (Fig. 2B). Consistent with these observations, we found that the amount of HSP90 immunoprecipitated GR was increased in liver extracts from Rev-erbα−/− compared to wild-type animals (Fig. 2C). Therefore, it is very likely that REV-ERBα modulates GR levels by binding to HSP90. In order to test whether REV-ERBα binding to HSP90 had an influence on the stability of GR, we treated NIH 3T3 cells with cycloheximide, a translation inhibitor, in presence of endogenous REV-ERBα (Fig. 2D, top, left side) and in presence of a plasmid leading to overexpression of REV-ERBα (Fig. 2D, top, right side). We found that the half-life of GR protein was reduced by overexpression of REV-ERBα and decreased from 10 h to 5.2 h (Fig. 2D, middle panel), indicating that overexpression of REV-ERBα affects the half-life of GR protein. Likewise, REV-ERBα overexpression affected the half-life of HSP90, reducing it from 8.7 h to 5.6 h (Fig. 2D, bottom panel). Hence, protein stability of GR appears to be related to HSP90 levels, which are directly or indirectly affected by REV-ERBα. We cannot exclude, however, the possibility that indirect effects stemming from changes in HSP90 levels can affect GR function through other HSP90 client proteins.

Fig. 1. GR protein, but not mRNA, is highly expressed in Rev-erbα−/− mice. (A) Western blots of liver extracts from wild-type (WT) and Rev-erbα−/− mice showing expression of GR and tubulin using their respective antibodies. Liver whole-cell lysates were extracted from wild-type and Rev-erbα−/− mice every 6 h, beginning at Zeitgeber time (ZT) 0 for one 24-h cycle. (B) Quantification of the GR signal normalized to tubulin (mean±s.d., n=3). *P<0.01 (two-way ANOVA). (C) Quantification of glucocorticoid receptor (Nr3c1) mRNA in the liver. Liver total RNA was extracted from wild-type and Rev-erbα−/− mice every 6 h, beginning at ZT0 for one 24-h cycle, and Nr3c1 transcripts were quantified (mean±s.d., n=3). (D) Quantification of glucocorticoid receptor (Nr3c1) mRNA in the liver. Liver total RNA was extracted from wild-type and Rev-erbα−/− mice every 6 h, beginning at ZT0 for one 24-h cycle, and Rev-erbα transcripts were quantified (mean±s.d., n=3). (E) Schematic representation of murine Nr3c1 promoter with its two REV-ERBα response elements (RORES). (F) Luciferase assay of Nr3c1::luc reporter constructs with Rev-erbα overexpression plasmids in NIH3T3 cells. Bmal1::luc was used as a positive control (mean±s.d., n=3). ***P<0.001 (one-way ANOVA with Bonferroni post-test).
REV-ERB\(\alpha\) affects diurnal subcellular localization of GR

In a next step, we wanted to know how the absence of REV-ERB\(\alpha\) affects nuclear and cytoplasmic localization of GR in liver cells. Immunohistochemistry on liver tissue from wild-type and Rev-erb\(\alpha\)\(^{-/-}\) mice harvested at ZT8 and ZT20 revealed profound alterations in the nuclear localization of GR that was dependent on both the presence of REV-ERB\(\alpha\) and the time of day (Figs 3 and 4). At ZT8, REV-ERB\(\alpha\) was strongly expressed in the nucleus of wild-type liver and absent in Rev-erb\(\alpha\)\(^{-/-}\) liver whereas HSP90 was expressed in both genotypes in the cytoplasm at both time points ZT8 and ZT20 (Fig. 3). At ZT20 REV-ERB\(\alpha\) was mainly in the cytoplasm in wild-type (at reduced levels as compared to nuclear REV-ERB\(\alpha\) at ZT8 as determined by the quantification, see below Fig. 5A) and absent in liver of Rev-erb\(\alpha\)\(^{-/-}\) mice (Fig. 3), illustrating the diurnal pattern of REV-ERB\(\alpha\) subcellular localization. Interestingly, this pattern was inverted for GR in wild-type animals (Fig. 4). GR was mainly cytoplasmic at ZT8 and absent in liver at ZT20. However, in Rev-erb\(\alpha\)\(^{-/-}\) mice, this diurnal pattern of subcellular localization of GR was absent and GR was present at both time points in the nucleus as well as in the cytoplasm (Fig. 4). In order to quantify these observations, we performed a cytoplasmic and a nuclear fractionation. REV-ERB\(\alpha\) was mainly nuclear at ZT8, whereas GR was mainly cytoplasmic at this time point in wild-type mice (Fig. 5A). The inverse was observed at ZT20 when REV-ERB\(\alpha\) was cytoplasmic and GR was nuclear. In Rev-erb\(\alpha\)\(^{-/-}\) mice, this diurnal pattern of localization of GR that was dependent on both the presence of REV-ERB\(\alpha\) and the time of day (Figs 3 and 4).

Diurnal interaction of GR and REV-ERB\(\alpha\) with HSP90 in liver and sites of interaction on HSP90

In order to determine the timing of GR and REV-ERB\(\alpha\) interaction with HSP90 in the liver, we collected total protein extracts from liver tissue around the clock from mice kept in a 12-h-light–12-h-dark cycle. Western blotting revealed constant levels of HSP90 and GR protein in extracts from livers of wild-type mice (Fig. 6A, input). REV-ERB\(\alpha\) was strongly expressed in the nucleus of wild-type mice (Fig. 5A). The inverse was observed at ZT20 when REV-ERB\(\alpha\) was cytoplasmic and GR was nuclear. In wild-type liver and absent in liver of Rev-erb\(\alpha\)\(^{-/-}\) mice (Fig. 5B). Taken together, these observations indicate that the presence of REV-ERB\(\alpha\) inversely correlates with a diurnal pattern in the subcellular distribution of GR.
upregulation of the expression of IκBα (Scheinman et al., 1995; Almawi and Melemedjian, 2002), a direct GR target (Fig. S1). To examine the effects of REV-ERBα on the NF-κB signaling pathway, we isolated primary hepatocytes at ZT8 and applied ethanol to these cells (Fig. 7), which is known to induce inflammatory reactions. In wild-type hepatocytes ethanol treatment led to an increase of phosphorylated IκBα (p-IκBα) and a reduction of unphosphorylated IκBα (Fig. 7A). As a consequence NF-κB translocated from the cytoplasm into the nucleus (Fig. 7B). In contrast, unphosphorylated IκBα accumulated in Rev-erbα−/− hepatocytes, probably as a consequence of increased GR levels in these animals (Fig. 7A,B), leading to reduced amounts of NF-κB in the nucleus. To test the above observations, we measured the levels of TNF secretion. Upon ethanol treatment TNF levels increased even in the presence of scrambled short hairpin RNA (shRNA) molecules (Fig. 7C). In contrast, ethanol did not induce TNF secretion in hepatocytes from Rev-erbα−/− animals. However, knocking-down GR or IκBα rescued, at least in part, ethanol-induced TNF secretion of Rev-erbα−/− hepatocytes (Fig. 7C). This indicates that REV-ERBα influences ethanol-induced TNF secretion through GR and IκBα in hepatocytes. For this process, however, glucocorticoids, GR ligands, are essential, because glucocorticoid-depleted Rev-erbα−/− hepatocytes display similar levels of p-IκBα, IκBα and NF-κB compared to wild-type hepatocytes (Fig. S2A; Fig. 2B). Therefore, TNF can be normally induced in glucocorticoid-depleted Rev-erbα−/− hepatocytes upon ethanol treatment (Fig. S2C).

Apparently, REV-ERBα affects GR targets, but does GR also affect REV-ERBα targets? We isolated wild-type hepatocytes and cultured them in presence of scrambled shRNA or GR shRNA. We observed that GR shRNA knocked down GR at the mRNA (Fig. S3)
and the protein levels (Fig. 7D), which led to an increase in REV-ERBα accompanied by a reduction in expression of its target BMAL1 (Fig. S3; Fig. 7D). This is consistent with previous findings (Preitner et al., 2002). Hence, GR can also affect the expression of at least some REV-ERBα targets.

**REV-ERBα and GR modulate the diurnal metabolic landscape**

REV-ERBα has been strongly implicated in the regulation of metabolism (Bugge et al., 2012; Cho et al., 2012). In order to test whether metabolic changes observed in livers of Rev-erbα−/− mice are related to GR, we compared genes changed in Rev-erbα−/− animals with genes regulated by GR (Phuc Le et al., 2005). If the hypothesis that REV-ERBα and GR influence expression of each other’s target genes were correct, we would expect to be able to identify changes in expression of direct GR targets in Rev-erbα−/− animals.

In a first step, we performed RNA sequencing of wild-type and Rev-erbα−/− livers at ZT8 and ZT20 and compared the data in order to identify transcripts that are altered at ZT8 but not at ZT20 (Fig. 8A). Because REV-ERBα is highly expressed at this time point, the changes in REV-ERBα are substantial between wild-type and Rev-erbα−/− animals, and, hence, the changes in GR nuclear localization would also be substantial. We identified 846 genes altered in expression in Rev-erbα−/− animals.

**Fig. 5. REV-ERBα and GR reciprocally influence their nuclear localization.** (A) Western blot of the cytoplasm and nuclear fraction of the liver of wild-type (WT) and Rev-Erbα−/− (Rev−/−) mice at ZT8 and ZT20. The lower panel shows the quantification of signals (mean±s.d., n=4). *P<0.05, ***P<0.001 (one-way ANOVA with Bonferroni post-test). (B) The levels of corticosterone in the plasma of wild-type (black bars) and Rev-Erbα−/− (white bars) mice revealed by ELISA (n=6, mean±s.d.).
more. The resulting 25 genes were submitted to ontology selection, and ordered according to biological process or molecular function. We identified two genes involved in immune system processes and 13 genes involved in metabolic processes, including *Bmal1*, a clock gene directly regulated by *REV-ERBα* (Preitner et al., 2002).

Then, we compared the 846 differentially expressed genes between *Rev-era−/−* and wild-type at ZT8 (Fig. 8A) with genes regulated by GR at around ZT4–ZT8 (Phuc Le et al., 2005). From these 846 genes, we found 50 genes in common with a list of genes altered in expression in livers after dexamethasone injection (Fig. 8B, overlap yellow and red circles; Table S1, green marked genes). Interestingly this list contained two genes involved in metabolic processes that were more than 1.5 times upregulated in *Rev-era−/−* liver (*Cdhn1a* and *Dde*). From the 846 genes, 16 genes were also present in a list of genes derived from a previous ChIP-on-Chip experiment at around ZT4–ZT8 (Phuc Le et al., 2005) using anti-GR antibodies, representing differences in promoter-binding after dexamethasone injection into livers (Fig. 8B, overlap red and green circles; Table S1, blue marked genes). In common between the expression analysis and the location analysis were eight genes that most likely represent direct GR targets altered in *Rev-era−/−* livers (Fig. 8B, overlap yellow, red and green circles; Table S1, yellow marked genes).

From these eight potential GR target genes, we verified three genes that displayed large changes by qRT-PCR, including alcohol dehydrogenase 1 (*Adh1*), a proven direct target of GR (Edenberg and Brown, 1992), glycine N-methyltransferase (*Gnmt*) and hepatic lipase C (*Lipc*) (Fig. 8C). We observed significant differences in the expression of these genes at ZT8 in *Rev-Erbα−/−* and wild-type livers (Fig. 8D, Western blots of primary cultured hepatocytes of wild-type (WT) and *Rev-Erbα−/−* mice). Cells were treated with GR or IκBα shRNA lentiviral particles. Scrambled shRNA lentiviral particles (sc) were used as a negative control. At 24 h after transduction, dexamethasone (1 μM) was added and cells were incubated for 24 h, and then the culture medium was changed for one with or without 100 mM ethanol (mean±s.d., n=3). *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA with Bonferroni post-test). (B) ELISA of TNF in primary cultured hepatocytes from wild-type and *Rev-Erbα−/−* mice. Cells were treated with GR or IκBα shRNA lentiviral particles. Scrambled shRNA lentiviral particles (sc) were used as a negative control. At 24 h after transduction, dexamethasone (1 μM) was added and cells were incubated for 24 h, and then the culture medium was changed for one with or without 100 mM ethanol (mean±s.d., n=3). *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA with Bonferroni post-test). (C) Western blot of primary cultured hepatocytes of wild-type mice. Cells were treated with GR or IκBα shRNA lentiviral particles. Scrambled shRNA lentiviral particles (sc) were used as a negative control. At 24 h after transduction, dexamethasone (1 μM) was added and cells were incubated for 24 h, and then the culture medium was changed for one with or without 100 mM ethanol (mean±s.d., n=3). *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA with Bonferroni post-test).
Both genes were upregulated in Rev-erbα−/− livers (Fig. 8D).

In order to test whether the verified genes in Fig. 8C,D are directly regulated by GR, we performed chromatin immunoprecipitation (ChIP) experiments. As control, we used the Per1 promoter, which contains a GR response element (GRE) (Reddy et al., 2009). Probes unrelated to the GRE for the Per1 promoter indicated the background signal for the ChIP experiments (Fig. 8E, top middle panel). Using specific probes flanking the GRE we observed a binding of GR to the GRE that was dependent on the time of day element in the Per1 promoter in wild-type, with low or no binding at ZT8 and binding at ZT20, but constant chromatin binding in liver derived from Rev-erbα−/− mice (Fig. 8E, top left panel). Similar GR binding was observed for Insig2a, Adh1, Gnmt and Lipc (Fig. 8E). These results indicate that these genes are regulated at least in part by GR, suggesting that the increase in gene expression observed in Rev-erbα−/− mice might stem from GR-related transcriptional mechanisms and not necessarily from lack of REV-ERBα-mediated transcriptional repression.

Taken together, these experiments support our hypothesis that REV-ERBα and GR influence each other, affecting expression of their respective target genes.

**DISCUSSION**

**REV-ERBα and GR influence each other**

GR (Nr3c1) and REV-ERBα (Nr1d1) both are nuclear receptors that modulate similar physiological pathways, including metabolism and inflammation (Tronche et al., 1998; Buckingham, 2006; Everett and Lazar, 2014). We previously observed that Nr3c1 mRNA expression is downregulated in the suprachiasmatic nuclei (SCN) of Rev-erbα−/− mice compared to wild-type animals (Schnell et al., 2014). This prompted us to test a potential relationship between REV-ERBα and GR. Interestingly, we found that, in the liver, GR protein in Rev-erbα−/− mice is upregulated compared to wild type (Fig. 1A,B). However, at the mRNA level, no differences in Nr3c1 expression between the two genotypes were observed (Fig. 1C). These findings indicate that GR in the liver is not regulated in the same manner as in the SCN and that regulation in
the liver most likely occurs at the post-transcriptional level (Fig. 1F).

In NIH 3T3 cells, we observed an inverse relationship between REV-ERBα and GR. The more REV-ERBα was expressed in the cells the less GR was detected (Fig. 2A). Given that GR interacts with heat-shock protein 90 (HSP90) before hormone-dependent activation (Kirschke et al., 2014), we wondered whether REV-ERBα would affect binding of GR to HSP90. We found that REV-ERBα interacts with HSP90 in a dose-dependent manner (Fig. 2B) influencing GR binding to HSP90 in an inverse fashion. This was confirmed in liver tissue from Rev-erbα−/− mice (Fig. 2C). The observation that the half-life of GR protein was shortened in presence of the REV-ERBα expression plasmid corroborated the above findings (Fig. 2D). Interestingly, however, REV-ERBα also affected the half-life of HSP90 (Fig. 2D). Hence, stability of GR could be mediated either directly or indirectly through modulation of stability of HSP90 upon changes in the amount of REV-ERBα. Taken together, we identified an inverse correlation between REV-ERBα and GR, which is most likely related to binding of these two nuclear receptors to HSP90.

Nuclear receptors regulate transcription and, hence, their activity depends on their localization to the nucleus. Using immunofluorescence and confocal microscopy we observed time-dependent subcellular distribution changes in GR and REV-ERBα in liver tissue (Figs 3, 4 and 5). In wild-type liver, GR was localized predominantly to the cytoplasm at ZT8 whereas it was nuclear at ZT20 (Fig. 4). In contrast, REV-ERBα was distributed between cytoplasm and nucleus in an inverse manner (Fig. 3). In the absence of REV-ERBα the time-dependent subcellular distribution changes in GR was not observed anymore (Fig. 4), indicating that REV-ERBα might play a role in this process. We do not know what the role of REV-ERBβ is, but it is probably not strong, because it cannot compensate for the loss of REV-ERBα. Other interpretation of the data is that REV-ERBα influences the subcellular distribution of GR through interaction with HSP90. However, we cannot exclude that REV-ERBα influences the machinery for translocation of GR into the nucleus. Corticosterone seems not to be a factor, though, because corticosterone levels in Rev-erbα−/− mice are not different compared to in wild type (Fig. 5B), as observed previously (Delezie et al., 2012).

**Sequential interaction of REV-ERBα and GR at different sites on HSP90**

In order to investigate the temporal profile of REV-ERBα and GR interaction with HSP90 we analyzed total liver extracts collected at 4-h intervals from ZT0 – ZT20 of wild-type and Rev-erbα−/− mice (Fig. 6A). We observed a sequential interaction of these two nuclear receptors with HSP90 between ZT0 and ZT12 with an overlap at ZT4. This indicates that the GR interaction with HSP90 disappeared upon the emergence of REV-ERBα interaction. Because the binding site of the two nuclear receptors to HSP90 appears not to be identical (Fig. 6B), a direct competition for the same binding site on HSP90 can be excluded. Possibly structural changes of HSP90 due to REV-ERBα binding might reduce the affinity of HSP90 for GR and release it. This possibility is supported by the observation that REV-ERBα protein levels start to increase at ZT4 with a maximum at ZT8 in liver tissue (Prettner et al., 2002), which coincides with the observed binding to HSP90 (Fig. 6A). Because most of the REV-ERBα protein appears to be nuclear at ZT8 (Fig. 3), the cytoplasmic HSP90-bound form of this nuclear receptor is most likely a differently modified or a truncated version. Evidence for a modified form of REV-ERBα can be seen in our overexpression experiment in Fig. 2D, where a double band of REV-ERBα protein was detected (Fig. 2D, 0 h after CHX treatment). It is known that REV-ERBα is phosphorylated and stabilized by GSK3β phosphorylation at serine residues 55 and 59 (Yin et al., 2006), and there might even be more modifications. Hence, post-translational modifications are important for the stability and might be important for the transport of REV-ERBα from the cytoplasm to the nucleus. Variants of REV-ERBα stemming from such processes are most likely dependent on the time of day, because at the time when REV-ERBα is predominantly cytoplasmic (ZT20) (Fig. 3) no interaction with HSP90 was observed (Fig. 6A).

Different forms of GR (splicing variants, translational initiation variants or post-translational modification variants; reviewed in Vandevyver et al., 2014) might also contribute to the inverse subcellular distribution observed in comparison to REV-ERBα. Evidence for this can be seen in Fig. 2C, where the input shows a double band for GR, which disappears after immunoprecipitation with HSP90 (Fig. 2C). This might affect REV-ERBα and its interaction with HSP90 and its subsequent subcellular localization. Taken together, it appears that the inverse subcellular localization we observed between GR and REV-ERBα is mechanistically very complex and not a simple competition between two nuclear receptors for binding to HSP90. Given that HSP90 is a very abundant protein in the cytoplasm, interaction with it is probably regulated by post-translational modification of its binding partners.

**REV-ERBα, GR and inflammation**

Diurnal variation of inflammatory function has been described (Castanon-Cervantes et al., 2010), but molecular mechanisms mediating its gating are unknown. Disruption of the circadian clock by deleting Bmal1 specifically in macrophages has been shown to remove all temporal gating of endotoxin-induced responses (Gibbs et al., 2012). This loss of circadian gating was coincident with suppressed Rev-erba expression. Given that REV-ERBα has been reported to modify expression of NF-κB-responsive genes in humans (Migita et al., 2004) and GR exerts anti-inflammatory action in part by antagonizing NF-κB through upregulation of IκBα (Scheinman et al., 1995; Almawi and Melemedjian, 2002), whose promoter contains GR-binding sites (Lim et al., 2015), we treated primary cultures of hepatocytes from control and Rev-erbα−/− mice with ethanol to activate this pathway (Fig. 7, Fig. S1). We found that this signaling pathway is inhibited in hepatocytes of Rev-erbα−/− mice, but knocking down GR or IκBα led to an almost normal induction of this signaling pathway as manifested by the induction of TNFα. Hence, REV-ERBα appears to be a link between the clock and immune function, most likely through its potential to interfere with the interaction of GR with HSP90, thereby gating nuclear entry of GR to ZT20 by an unknown and probably indirect mechanism (Fig. 4). This provides a rational for the observation that in mice with a bronchiole-specific deletion of Bmal1, circadian binding of GR at the Cxcl5 locus is disrupted (Gibbs et al., 2014). In these animals REV-ERBα expression is strongly reduced, and the presence of GR in the nucleus would not be gated anymore. Accordingly, Cxcl5 would not be rhythmically, but rather constantly expressed, which has been observed in lung tissue of these animals (Gibbs et al., 2014). Taken together, this indicates that gating of GR to the nucleus involving REV-ERBα most likely does not only occur in liver but in other tissues such as the lung as well.

**REV-ERBα, GR and metabolism**

Metabolism is affected in both Rev-erba and GR liver-specific knockout mice (Opherk et al., 2004; Bugge et al., 2012; Cho et al.,
2012). Mice lacking GR in the liver display faster phase-resetting of the liver clock upon restricted feeding (Le Minh et al., 2001), and Rev-erbα−/− mice exhibit a reduction in food anticipatory behavior (Delezie et al., 2012). This illustrates that metabolism is modulated by these two nuclear receptors.

Our data support this view. For example, alcohol dehydrogenase 1 (Adh1) expression is altered in the liver of Rev-erbα−/− mice (Fig. 8C) and is regulated by GR, as evidenced by ChIP (Fig. 8E), illustrating the potential consequence of lack of REV-ERBα on GR expression at ZT8. Similar effects were observed for glycerol N-methyltransferase (Gnmmt) and hepatic lipase C (Lipc), enzymes involved in liver detoxification and lipid metabolism, respectively. Although these genes appear to be regulated by GR (Fig. 8E), the pathways they are involved in are also altered in Rev-erbα−/− mice (Le Martelot et al., 2009; Bugge et al., 2012; Cho et al., 2012). Most interestingly, Insig2 an oxysterol sensor, that sequesters sterol-regulatory-element-binding proteins to the endoplasmic reticulum and thereby indirectly modulates fatty acid and cholesterol metabolism, has been identified as a bona fide REV-ERBα target (Le Martelot et al., 2009). Our data confirm the change of Insig2α gene expression in Rev-erbα−/− mice (Fig. 8D) with the twist that this gene can be regulated by GR, as evidenced by ChIP (Fig. 8E). These results suggest a close relationship between REV-ERBα- and GR-mediated transcriptional regulation.

In summary, we show in this study that REV-ERBα influences the subcellular distribution of the GR protein by an unknown mechanism. As a consequence these two nuclear receptors influence the transcriptional potential of each other leading to a temporal segregation of GR target gene expression. This illustrates the complex cross-regulation between nuclear receptors, providing an explanation for off-target effects of drugs modulating specific nuclear receptor functions.

MATERIALS AND METHODS

Animals

Animal care and handling was performed in accordance with the guidelines of the Schweizer Tierschutzgesetz (TSchG, SR455) and the declaration of Helsinki authorized by the Office Vétérinaire Cantonal de Fribourg. Rev-erbα-knockout mice (Petrinet et al., 2002, 129/C57BL/6 mixed background) were obtained from heterozygous breeding pairs originally provided by Prof. Ueli Schibler (Department of Molecular Biology, University of Geneva, Geneva). Two- to four-month-old male mice were used for experiments and wild-type mice served as controls. Animals were kept under 12 h light and 12 h dark conditions with food and water ad libitum.

Cell culture

NIH3T3 mouse fibroblast cells (ATCC®CRL-1658™) were maintained in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal calf serum (FCS) and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO2. NIH3T3 cells were treated with 100 μM cycloheximide after transfection containing either empty vector or PSCT1-Rev-erbo. Cells were harvested 0, 2, 4 and 6 h after treatment.

Quantitative real-time PCR

Total RNA was extracted from snap-frozen liver tissue using RNA- Bee (AMS Biotechnology), according to the manufacturer’s instructions. Liver RNA was precipitated in 4 M LiCl to remove glycogen, and was purified further by phenol:chloroform extraction and ethanol precipitation. Single-stranded DNA (ssDNA) complementary to the RNA starting from hybridized random hexamer primers was synthesized with SuperScript II (Life Technology Corporation) according to the manufacturer’s instructions. SYBR green fluorescence-based qRT-PCR was performed for RNA quantification (KAPA SYBR FAST Universal, KAPA Biosystems, RotorGene 6000, Corbett Research). All RNA samples were normalized to Gapdh. Primers are listed in Table S2.

Luciferase reporter assays and transfection

A 1756-bp fragment of the mouse Nr3cl promoter region (~1750 to +6 bp from the transcription start site containing RORE elements) was cloned into the pGL3 basic vector (Promega) using the following primers: 5’-CGGT-ACCTGAAGTCGTGCTCCTAAC-3’ (sense primer) and 5’-CCTCGAGG-TCTCCAAGAAACAC-3’ (anti-sense primer). Transfection and luciferase reporter assays were performed with NIH3T3 cells according to Schnell et al. (2014). The Bmal1 promoter region cloned into pGL3 were used as positive controls.

Western blotting analysis

Protein of cultured cells and liver tissue was extracted using RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate containing protease and phosphatase inhibitors). Protein samples (40 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose (Amerham Protran Supported 0.45 NC, GE healthcare). After blocking with 0.5% dry milk in PBS-Tween 0.1%, the membranes were probed with antibodies against GR (1:500, Santa Cruz Biotechnology, sc-1004), HSP90α/β (F-8) (1:1000, Santa Cruz Biotechnology, sc-13119), REV-ERBα (1:200, Santa Cruz Biotechnology, sc-100910), BMAL1 (1:500, Rippinger and Schibler, 2006), NF-κB p65 (1:200, Santa Cruz Biotechnology, sc-109), IκBα (1:200, Santa Cruz Biotechnology, sc-371), phosphorylated (p-)IκBα (1:200, Santa Cruz Biotechnology, sc-8404), tubulin (1:1000, Abcam, ab 15246) or LaminB1 (1:500, Santa Cruz Biotechnology, sc-30264) overnight at 4°C. Anti-rabbit-IgG, mouse-IgG and goat-IgG antibody conjugated to horseradish peroxidase (HRP) was used as a secondary antibody. Detection of the immune complexes was performed using Western Bright Quantum system (Advansta) and quantification was done with the Quantity One analysis software (BioRad).

Pulldown assay with HSP90 fragments

GST-fused recombinant HSP90α proteins were expressed in an E. coli BL21 strain [plasmids: GST-Hsp90 N(9-236), GST-Hsp90M(272-617), and GST-Hsp90 C(626-732), Addgene 22481, 22482 and 22483, respectively]. Proteins were purified to homogeneity with glutathione–agarose beads for 2 h at 4°C. The beads were then incubated overnight at 4°C with purified GR and REV-ERBα both containing a His-tag (GR-LBD: pET15Avi6HT-Nr3c1 DNASU plasmid no. HsCD00651869; Rev-erbα: pET15bxRev-erbo). Subsequently, elution with 10 mM reduced glutathione took place for 15 min at room temperature. Elution was stopped by adding Laemmli buffer and samples were loaded onto the gel after 10 min at 95°C.

Preparation of cytoplasm and nuclear extracts from primary cultured hepatocytes

The isolated hepatocytes nuclear extracts were washed with PBS and then were homogenized in ice-cold homogenization buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 0.1 mM DTT and 0.1% NP40) containing protease inhibitor (1 μg/ml). The homogenates were incubated on ice for 30 min and nuclei were collected by centrifugation at 800 g for 5 min at 4°C. Nuclei were resuspended in extraction buffer (20 mM HEPES pH 7.9, 10% glycerol, 400 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 0.1% NP40) containing protease inhibitor. After incubation on ice for 1 h, cell debris and DNA was removed by centrifugation at 21,000 g for 10 min at 4°C. Extracts were aliquoted and stored at −80°C.

Co-immunoprecipitation

For co-immunoprecipitation experiments with cultured cells and mouse whole-liver extracts, 400 μg of whole extracts were incubated with rotation overnight at 4°C with the indicated antibody and captured with protein–A-agarose beads (Roche Applied Science) for 4 h at 4°C. The beads were washed four times with RIPA buffer. After washes, the samples were loaded onto the gel after 10 min at 95°C.
resuspended in 4× SDS protein sample buffer (40% glycerol, 240 mM Tris-HCl pH 6.8, 8% SDS, 0.04% Bromophenol Blue, 5% β-mercaptoethanol) and subjected to SDS-PAGE.

**Mouse hepatocyte isolation**

Hepatocytes were isolated from livers of Rev-erbα−/− and wild-type mice using collagenase perfusion with modifications. The inferior vena cava was cannulated and the liver was first perfused in situ with a Hanks' buffer salt solution (HBSS), pH 7.4 (4 ml/min, 37°C for 10 min), followed by perfusion with HBSS containing 1 mM Ca²⁺ and Mg²⁺ and 0.01% collagenase type 1 (Thermo Fisher Scientific), pH 7.4, for 10 min. The liver was removed and then gently minced in HBSS containing 1 mM Ca²⁺ and Mg²⁺ and 0.01% collagenase type 1, pH 7.4. The liver cell suspension was then filtered with Falcon cell strainers (70 mm; Becton Dickinson, Bedford, MA) and centrifuged at 500 g for 10 min. Cells were plated on a collagen-coated plate (Thermo Fisher Scientific, 6×10⁵ cells/well) in DMEM containing 10% FCS and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. After an initial 5-h attachment period, cultures were washed with PBS and then fresh culture medium.

**Immunohistochemistry**

Animals used for the immunohistochemistry were killed at ZT8 and ZT20. Brains were perfused with 0.9% NaCl and 4% paraformaldehyde (PFA). Perfused brains were cryoprotected by 30% sucrose solution, and sectioned (15 μm) using a cryostat. Sections chosen for staining were washed three times with 1× TBS and 2× SSC (pH 7, 0.3 M NaCl,0.03 M tri-Na-citrate). Antigen retrieval was performed with 50% formamide and 2× SSC by heating to 65°C for 60 min. Later, sections were washed in 2× SSC and in 1× TBS pH 7.5 (0.1 M Tris-HCl, 0.15 M NaCl), before blocking them for 2 h in 10% normal donkey serum sterile (NDS, ab138579, abcam, UK), 0.1% Triton X-100 and 1× TBS at room temperature. After the blocking, primary anti-Rev-Erbα antibody (ab174309, abcam, 1:100), anti-GR (Santa Cruz Biotechnology, sc-1004, 1:50) and anti-HSP90 antibody (Santa Cruz Biotechnology, sc-13119, 1:50), diluted in 1% normal donkey serum (NDS), 0.1% Triton X-100 and 1× TBS were added to the sections and incubated at 48 h at 4°C. Sections were washed with 1× TBS and incubated with the appropriate fluorescent secondary antibodies diluted 1:500 in 1% NDS, 0.1% Triton X-100 and 1× TBS for 3 h at room temperature [Alexa Fluor® 488-AffiniPure donkey anti-rabbit IgG (H+L), 711-545-152, and Alexa Fluor® 647-AffiniPure donkey anti-mouse IgG (H+L), 715-605-150; Jackson Immuno Research]. After washing with 1× TBS, nuclei were counterstained with DAPI (300 nM) for 15 min. Finally, the tissue sections were incubated in objective 20× a resolution of 1024×1024, 300 μm through whole section with frame average of 3. Images were processed with LAS AS software from LEICA.

**Preparation of cytoplasm and nuclear extracts from liver**

Small piece of frozen liver was homogenized in ice-cold homogenization buffer (100 mM Tris-HCl, 10 mM DTT). The homogenates were incubated on ice for 10 min and centrifuged at 2900 g for 5 min at 4°C. Pellets were resuspended in 200 μl complete cytoplasm lysis buffer (10 mM EDTA, 10 mM Hepes, 0.2 mM NaF, 0.2 mM Na Vanadate, 2 mM DTT, and 0.2% Triton X-100) containing protease inhibitor (Roche) and centrifuged at 2900 g for 5 min at 4°C. Supernatant was collected as cytoplasm extracts. After three washes with complete cytoplasm lysis buffer, nuclei were resuspended in 100 μl NDB buffer (20% glycerol, 20 mM Hepes, 0.2 mM EDTA, 0.4 mM Na Vanadate, 2 mM DTT and 100 mM KCl) containing protease inhibitor and 100 μl NUN buffer (2 M urea, 600 mM NaCl, 2% NP-40, and 50 mM Hepes) were added. After incubation on ice for 30 min, cell debris and DNA was removed by centrifugation at 15,700 g for 30 min at 4°C. Supernatant was collected as for nuclear extracts.

**Plasma corticosterone measurement**

Under gaseous isoflurane anesthesia, blood samples were collected from the tail vein at different time points (ZT8 and ZT20). Plasma was recovered by centrifuging samples at 1500 g for 10 min in an Eppendorf centrifuge. Plasma corticosterone levels were measured with the Corticosterone EIA kit (ADI-900-097, Enzo Life Sciences) according to the manufacturer’s instructions.

**Knockdown of GR and IkBα, ethanol treatment and measurement of TNF**

At 24 h after seeding cells, shRNA lentiviral particles (GR; sc-35506-V, IκBα; sc-29361-V, Santa Cruz Biotechnology) were used for knocking down GR and IκBα according to the manufacturer’s instructions. Knockdown efficiency was assessed at 48 h post transduction by western blotting. Scrambled shRNA lentiviral particles (sc-108080, Santa Cruz Biotechnology) was used as a negative control. At 24 h after transduction, dexamethasone was added to a final concentration of 1 μM. After 24 h, the culture medium was changed for one with or without 100 nM ethanol. After 24 h, the medium was collected and the levels of TNF were measured with the TNF alpha ELISA Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cells were washed twice with phosphate buffer, scraped and subjected to protein extraction.

**RNA extraction and library construction**

Liver samples were immediately flash frozen in liquid N₂ and stored at −80°C. RNA was extracted using NucleoSpin RNA (Machery-Nagel, Düren, Germany) according to the instructions of the manufacturer. The quality of the RNA samples was analyzed with a spectrophotometer, agarose gel electrophoresis and reverse-transcription-PCR. Library construction starting from the poly(A)-tail and multiplexing was performed according to the instructions of the manufacturer (Illumina). The samples were organized as follows: three replicates (1-WT, 2-WT, 3-WT) correspond to genotype WT at ZT8; three replicates (4-Rev, 5-Rev, 6-Rev) correspond to genotype Rev-erbα−/− at ZT8; three replicates (7-WT, 8-WT, 9-WT) correspond to genotype WT at ZT20; three replicates (10-Rev, 11-Rev, 12-Rev) correspond to genotype Rev-erbα−/− at ZT20.

For the experiment, complementary DNA (cDNA) libraries were barcoded using Illumina primers and loaded onto one lane of an IlluminaHS2000 machine. cDNA libraries were diluted and loaded onto each lane. The samples were sequenced for a maximum sequencing length of 75 bp.

**Analysis of RNA-Seq data sets**

Sequences were aligned to the mouse genome (UCSC version mm10 database). Numbers of the sequences obtained for each library can be found in Table S3. Sequences (fastq format) were mapped with Tophat (Trapnell et al., 2009) (http://amigo.geneontology.org/amigo). Analysis of GO data was done using term enrichment analysis, finding significant shared GO terms or parents of those GO terms to help discover what input genes might be in common (Mi et al., 2013).

**Pathway analysis**

The analysis was performed using official web-based set of tools for searching and browsing the Gene Ontology database AmiGO 2 (Carbon et al., 2009) (http://amigo.geneontology.org/amigo). Analysis of GO data was done using term enrichment analysis, finding significant shared GO terms or parents of those GO terms to help discover what input genes might be in common (Mi et al., 2013).

**Chromatin immunoprecipitation**

ChiP was performed as described previously (Ripperger and Schibler, 2006) using an anti-GR antibody at a dilution of 1:25 (M-20, sc-1004, Santa Cruz Biotechnology) was used as a negative control. At 24 h after transduction, dexamethasone was added to a final concentration of 1 μM. After 24 h, the culture medium was changed for one with or without 100 nM ethanol. After 24 h, the medium was collected and the levels of TNF were measured with the TNF alpha ELISA Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cells were washed twice with phosphate buffer, scraped and subjected to protein extraction.
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