Glucocorticoid Receptor β Induces Hepatic Steatosis by Augmenting Inflammation and Inhibition of the Peroxisome Proliferator-activated Receptor (PPAR) α**

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Glucocorticoids (GCs) regulate energy supply in response to stress by increasing hepatic gluconeogenesis during fasting. Long-term GC treatment induces hepatic steatosis and weight gain. GC signaling is coordinated via the GC receptor (GR) GRα, as the GRβ isoform lacks a ligand-binding domain. The roles of the GR isoforms in the regulation of lipid accumulation is unknown. The purpose of this study was to determine whether GRβ inhibits the actions of GCs in the liver, or enhances hepatic lipid accumulation. We show that GRβ expression is increased in adipose and liver tissues in obese high-fat fed mice. Adenovirus-mediated delivery of hepatic GRβ overexpression (GRβ-Ad) resulted in suppression of gluconeogenic genes and hyperglycemia in mice on a regular diet. Furthermore, GRβ-Ad mice had increased hepatic lipid accumulation and serum triglyceride levels possibly due to the activation of NF-κB signaling and increased tumor necrosis factor α (TNFα) and inducible nitric-oxide synthase expression, indicative of enhanced M1 macrophages and the development of steatosis. Consequently, GRβ-Ad mice had increased glycogen synthase kinase 3β (GSK3β) activity and reduced hepatic PPARα and fibroblast growth factor 21 (FGF21) expression and lower serum FGF21 levels, which are two proteins known to increase during fasting to enhance the burning of fat by activating the β-oxidation pathway. In conclusion, GRβ antagonizes the GC-induced signaling during fasting via GRα and the PPARα–FGF21 axis that reduces fat burning. Furthermore, hepatic GRβ increases inflammation, which leads to hepatic lipid accumulation.

A diet high in fat can lead to obesity and lipid accumulation in the liver, which increases proinflammatory cytokine production and the development of non-alcoholic fatty liver disease (NAFLD). Also, NAFLD leads to peripheral tissue insulin resistance, possibly through a reduction of insulin clearance in the liver due to the build-up of lipids. NAFLD is characterized by hepatic fat accumulation that, when coupled with another “hit,” such as increased oxidative stress, insulin resistance, or inflammation can lead to the development of non-alcoholic steatohepatitis (NASH) (1). The progression of hepatic steatosis may only represent the initial phase of several distinct deleterious pathways, and a “two-hit” theory has been used to explain the progression from NAFLD to NASH. The “first” hit is considered to be the accumulation of lipids in the liver. The “first hit” increases the vulnerability of the liver to other factors that may contribute to the “second hit” and promote hepatic injury and inflammation (2).

Interestingly, long-term glucocorticoid (GC) treatment, which is a known anti-inflammatory, induces hepatic steatosis and insulin resistance (3). The GC receptor (GR), is a complex single copy gene that is alternatively spliced to give rise to different isoforms α, β, γ, A, and P (4). Due to direct binding, the GRα isoform is the protein responsible for the actions of GCs. GRβ, however, has a truncated helix 12 and is missing the ligand binding pocket known to bind to GCs (3). The known function of GRβ is to act as a dominant-negative antagonist to GRα (3, 5, 7–9). We have previously shown that GRβ mRNA increased from fasting and refeeding in the livers of mice (8). Similarly, Dubois et al. (10) demonstrated that the rat GRβ has a physiological role in the liver and rat GRβ mRNA expression is increased in animals that have chronically elevated plasma insulin concentrations. Recently, He et al. (11) showed in a

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‡ The abbreviations used are: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; GC glucocorticoid; GYS2, glycogen synthase 2; iNOS, inducible nitric-oxide synthase; GR, GC receptor; HFD, high-fat diet; GSK3β, glycogen synthase kinase 3β; AAV, adenoviral-associated virus; Ad, adenovirus; PTEN, phosphatase and tensin homolog deleted on chromosome 10; AMPK, AMP-activated protein kinase; Glut1, glucose transporter 1.
mouse with hepatic-specific GR knock-out and delivery of human GRβ-regulated gluconeogenesis and inflammation in liver. These studies suggest that GRβ may have a role in the regulation of hepatic lipid storage and peripheral insulin resistance. However, the functions of the GR isoforms in the development of hepatic steatosis or insulin resistance remains to be clarified.

During fasting, lipids that are taken up by the liver are a product of GC-induced lipolysis from adipose tissue (3). Typically, the glycerol released in adipose during fasting is used for gluconeogenesis in the liver for the production of new sugars from non-hexose substrates, which allows for the maintenance of blood glucose. The overstimulation of lipolysis or saturation of the blood with lipids from the diet leads to the accumulation of hepatic fat, de novo lipid synthesis, and inflammation. Lipid storage and the burning of fat are mediated by fat sensing nuclear receptors, PPARγ and PPARα, where PPARγ stores fat and PPARα burns lipids by β-oxidation. Hepatic PPARα increases the fat burning and glucose-lowering hormone, fibroblast growth factor 21 (FGF21), which causes a dramatic decrease in lipid accumulation in whole animals and sensitization to peripheral insulin signaling (12–19). A reduction in PPARα in the liver causes the development of fatty liver and inflammation (12, 19). The GRβ isoform has been shown to increase immune cell proliferation by inhibiting the anti-inflammatory actions of GCs (3, 20), as well as increase inflammatory pathways in liver (11). Potentially, GRβ may be involved in the initial phases of the development of NAFLD.

In this investigation, we found that elevated hepatic GRβ increased lipid accumulation on a regular fat diet leading to elevated plasma glucose. Furthermore, we show that GRβ decreased hepatic PPARα expression resulting in a reduction of the hepatic FGF21 mRNA and plasma levels. Our data show that GRβ may serve as the first hit in the progression of NAFLD.

**Results**

**GRβ Expression Increases in Response to HFD**—Acute elevations in GC promote adipose tissue lipolysis and hepatic gluconeogenesis, which is orchestrated by GRα. It has been proposed that obesity is a state of GC resistance that results in lipogenesis in adipose and the liver, leading to fatty liver disease (3). A probable mechanism for GC resistance in response to HFD is increased expression of GRβ. We have shown that GRβ mRNA is increased during adipogenesis, whereas GRα expression was unchanged (21). In Fig. 1, A and B, we show that a HFD indeed increased GRβ mRNA in adipose and liver, and this was accompanied by an elevation in transcript levels of the pro-inflammatory cytokine TNFα. Neither GR isoform nor TNFα mRNA was altered in skeletal muscle in response to HFD (Fig. 1C), indicating that adipose and liver dysfunction may precede changes in skeletal muscle.

**Overexpression of GRβ in Mice Livers**—Our data thus far indicate that under conditions of a HFD, GRβ transcript levels increased in liver and adipose tissues, which correlated with elevated TNFα expression, an inflammatory cytokine that is associated with hepatic inflammation and fatty liver disease (22, 23). To determine the role of GRβ in hepatic lipid accumulation we constructed an adenovirus with mouse GRβ cDNA (GRβ-Ad) and vector (vec-Ad). We next used the GRβ-Ad to overexpress GRβ in the liver to determine whether elevated GRβ expression is sufficient to induce hepatic steatosis. We infected the mice with GRβ-Ad or vec-Ad for 5 days on a regular fat diet. Overexpression of GRβ was successfully targeted to the liver, without affecting adipose or muscle (Fig. 2, A–C). Importantly, GRα protein and mRNA levels were not affected in liver, muscle, or adipose (Fig. 2, A–C).

**Overexpression of Hepatic GRβ Increases Lipid Accumulation**—Liver function is paramount in maintaining fat and glycogen storage, which also modulates normal circulating glucose levels. Recently Robert et al. (24) showed that in obese ob/ob mice the GR-Gilz pathway was suppressed in Kupffer liver cells, which caused lipid accumulation, suggesting that GRα-directed paths are preventive in hepatic steatosis. He et al. (11) showed that adenoviral-associated virus (AAV) delivery of human GRβ in mouse livers caused an increase in inflammatory pathways. However, they did not measure hepatic lipid content. Therefore, we wanted to determine whether increasing GRβ in the livers of normal mice would cause an increase in hepatic lipid accumulation and possibly inflammatory markers. The histological assessment revealed increased Nile red staining for lipid accumulation in the liver of mice overexpressing GRβ (Fig. 3A). Moreover, hepatic triglycerides (p < 0.05) (Fig. 3B) and serum triglycerides (p < 0.0001) (Fig. 3C) were markedly elevated in liver overexpressing GRβ. Also, fatty acid synthase protein, but not mRNA, expression was significantly (p < 0.05) elevated in the liver of GRβ-Ad mice compared with vec-Ad, indicating increased de novo fat synthesis (Fig. 3D). Insulin resistance in the liver causes an increase in hepatic glucose production that results in hyperglycemia. Hepatic lipid accumulation is one mechanism underlying this response (25, 26). Here we show that mice overexpressing GRβ in the liver have elevated fasting glucose levels compared with vector-treated animals (Fig. 3E).

**Hepatic Overexpression of GRβ Suppresses Glycogen Storage**—The liver serves as a primary reservoir for glycogen, which is used to maintain blood glucose levels during fasting. In livers overexpressing GRβ, glycogen content was reduced by 21%, but not significant, compared with Ad-vector-treated mice, which may be due to the reduction in expression of glycogen synthase 2 (Gys2) (Fig. 4A). Gys2 stores glucose as glycogen in the liver for use during times of fasting and GSK3β phosphorylates Gys2 to reduce glycogen storage. Phosphorylation of serine 9 of GSK3β inhibits kinase activity, which typically occurs during feeding. The phosphorylation of serine 9 of GSK3β was significantly lower (p < 0.01) in GRβ-Ad mice indicating activation (Fig. 4B). Additionally, the expression of the gluconeogenic genes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase was reduced in liver overexpressing GRβ (Fig. 4C). However, Foxo1, which also regulates gluconeogenesis, was unchanged. Taken together, this indicates that GRβ causes hepatic lipid accumulation and possibly interferes with glycogen storage in long-term high GRβ levels.

We previously demonstrated that GRβ regulates cell growth through an Akt1-PTEN dependent signaling (9). Here we show that hepatic PTEN is suppressed when GRβ is overexpressed in the liver (Fig. 4D), consistent with our previous findings (26).
However, Akt1 expression was not altered. On the other hand, GRβ reduced hepatic Akt2 expression. Akt2 has been shown to regulate metabolic function, particularly glucose homeostasis (27). Akt increases glycogen storage by inhibition of GSK3β through phosphorylation of serine 9. GRβ may serve to increase lipid accumulation during times of fasting and reduce glycogen storage to inhibit gluconeogenesis and hepatic glucose production.

**Hepatic Overexpression of GRβ Increases NF-κB Activity**—GRβ has recently been shown to cause inflammation in the liver (11). However, the role of GRβ in the regulation of M1 proinflammatory or M2 anti-inflammatory macrophages or the direct effect of GRβ on NF-κB phosphorylation or transcriptional activity is unknown. The NF-κB signaling pathway contributes to the regulation of inflammation, particularly by increasing the production of TNFα. Also, NF-κB expression is increased in livers with inflammation and steatosis (22, 28). In Fig. 5A we show that NF-κB serine 536 phosphorylation is elevated and the protein and mRNA expression of IκBα, an inhibitor of NF-κB, was suppressed in livers overexpressing GRβ. No significant change was observed in F480 total macrophage marker expression (Fig. 5B). However, GRβ overexpressing livers had higher expression of proinflammatory M1 macrophage markers TNFα and inducible nitric-oxide synthases (iNOS), and reduced anti-inflammatory M2 macrophage markers arginase 1 (Arg-1) and FIZZ1 (Fig. 5C). Even though there was no change in total macrophage marker, these results suggest that GRβ enhanced proinflammatory macrophages and reduced...
anti-inflammatory M2. Furthermore, we demonstrated that 
GRβ, but not GRα, increased the promoter activity of NF-κB (Fig. 5, D and E), indicating that overexpression of GRβ in the liver enhances NF-κB activity in the pathogenesis of hepatic steatosis. Last, the promoter activity of the tumor suppressor PTEN was decreased in response to GRβ, which is consistent with our previous findings (7, 9). NF-κB has also been shown to reduce PTEN expression (29). Here we show that NF-κB and GRβ together reduced the PTEN promoter more significantly (p < 0.05) than either transcription factor alone. In Fig. 5C, we show that GRβ significantly increased NF-κB activity, and this effect was lost with GRβ alone. GRα had no effect on NF-κB activity at the NF-κB-luc promoter but did significantly (p < 0.05) decrease the promoter without overexpression of p65. Interestingly, GRα attenuated NF-κB suppression of the PTEN promoter and increased the PTEN-luc. The overexpression of GRβ in mice livers caused a shift in macrophages to the pro-inflammatory M1 population, which combined with enhanced NF-κB activation leads to hepatic lipid accumulation and steatosis.

**Hepatic GRβ Suppresses PPARα-mediated Signaling**—PPARα is a nuclear receptor that has been shown to attenuate hepatic lipid accumulation, and is reduced in obesity (9, 12, 30–34). Also, PPARα is increased in the liver during fasting and reduced during feeding (35). Interestingly, GRβ overexpression in mice livers decreased PPARα protein and mRNA expression on a normal diet (Fig. 6, A and B). PPARα has been shown to increase genes in the β-oxidation pathway to reduce lipid accumulation. Also, PPARα increases the hormone fibroblast...
growth factor 21 (FGF21) in the liver to be excreted to affect glucose and lipid storage in adipose and liver (12, 14, 17, 19, 36–39). FGF21 is increased by PPARα during fasting, which elevates AMP-activated protein kinase (AMPK) and glucose transporter 1 (Glut1) for energy metabolism and glucose usage (15, 17–19, 36, 38, 40). The FGF21 protein and mRNA were decreased in GRα-Ad livers and serum (Fig. 6, A and B). Additionally, the FGF21-regulated gene, Glut1, was reduced in livers with overexpressed GRα. Interestingly, Glut1 and AMPK mRNA expression, but not FGF21, was also decreased in adipose tissue of the GRα-Ad mice, although GRα expression was not increased. The reduction in serum FGF21, which originates from the liver (40), also caused decreased signaling in adipocytes.

We then overexpressed GRβ or GRα versus PPARα and measured the activity at the minimal PPAR promoter PPRE-3tk-Luc to determine whether they regulate PPARα transcriptional activity. GRβ significantly decreased PPARα transcriptional activity with vehicle (p < 0.01) and PPARα agonist WY-14,643 by over 50% (p < 0.0001) (Fig. 7A). Conversely, GRα overexpression caused a significant (p < 0.01) increase in PPARα transcriptional activity. Glucocorticoids have been shown to regulate FGF21 expression in a feed-forward loop (41). To determine whether GRβ or GRα can regulate FGF21 expression, we measured luciferase activity of the FGF21 promoter construct (FGF21-Luc) with GRβ or GRα overexpressed. Not surprisingly, GRα significantly increased FGF21 promoter activity (Fig. 7B). However, GRβ alone had no effect. To determine whether GRβ or GRα regulate PPARα transcriptional activity at the FGF21 promoter, we treated cells overexpressing GRβ or GRα with WY-14,643. Interestingly, GRβ inhibited PPARα activity by 26% at the FGF21 promoter (p < 0.001), but GRα had no effect (Fig. 7C).

**Discussion**

We show in this study that GRβ increases lipid storage in the liver, and functions as the precursor for hepatic inflammation, which may act as the first hit in the progression of NAFLD. Inflammation in the liver serves as a precursor for several deleterious events that lead to hepatic lipid accumulation and NAFLD (19, 34, 42–45). The liver does secret TNFα, which causes infiltration of the immune system and is considered one of the many hits in the first stages of NAFLD (46). To further determine the specific role of GRβ in the development of
hepatic steatosis, we developed a model of increased hepatic expression using an adenoviral approach. Recently, He et al. (11) showed that an AAV-mediated delivery of human GR/H9252 in mice caused increased inflammation. Furthermore, Warrier et al. (45) demonstrated that mice with glucocorticoid resistance are susceptible to diet-induced hepatic steatosis and inflammation. In our murine model, hepatic-specific viral overexpression of mouse GRβ resulted in the augmentation of liver fatty acid production as evidenced by increased serum and hepatic triglycerides, and alterations in proteins associated with fatty acid synthesis and marked hepatic steatosis, which occurred in a very short (5 day) time frame and on a regular fat diet. In addition, hepatic overexpression of GRβ resulted in hyperglycemia without significant changes in insulin levels, which sug-

FIGURE 4. GRβ activates GSK3β and suppresses gluconeogenic genes. A, glycogen content and glycogen synthase kinase 2 (GYS2) expression in livers of GRβ-Ad and vec-Ad mice. *, p < 0.05 (versus vec-Ad mice) (± S.E.; n = 7). B, GRβ overexpression decreases serine 9 phosphorylation of GSK3β. **, p < 0.01 (versus vec-Ad mice) (± S.E.; n = 7). C, gluconeogenic genes were decreased in livers of GRβ-Ad mice compared with vec-Ad. **, p < 0.01 (versus vec-Ad mice) (± S.E.; n = 7). D, real-time expression of PTEN, Akt1, and Akt2 in livers GRβ-Ad and vec-Ad mice. *, p < 0.05; **, p < 0.01 (versus vec-Ad mice) (± S.E.; n = 6).
suggests alterations in gluconeogenesis or reduced hepatic insulin signaling due to increased lipids. All of these factors are altered in the obese and those with metabolic disorders.

Our results also demonstrate that diet-induced obese mice have a selective increase in GRβ expression in adipose and the liver without any changes in the levels of GRα in these tissues. The increase in GRβ expression was also associated with an elevation in the levels of the pro-inflammatory cytokine TNFα. In addition to our group, others have shown that TNFα and proinflammatory cytokines increase GRβ expression (8, 47–49). TNFα levels are known to be higher in the obese (50, 51). However, if TNFα induces GRβ in obesity is unknown. We did show in this investigation that overexpression of GRβ in mouse livers caused an increase in TNFα and iNOS, which are M1 proinflammatory macrophage markers, with no change in total macrophages (F480). GRβ may drive the infiltration of the immune system in the liver, which is a known characteristic of the onset of NAFLD.

FIGURE 5. GRβ increases NF-κB activity and M1 proinflammatory macrophages. A, representative Western blot and densitometry of serine 536 phosphorylation of NF-κB and total NF-κB as well as IκBα mRNA in livers of GRβ-Ad and vec-Ad mice. *, p < 0.05; **, p < 0.01 (versus vec-Ad mice) (± S.E.; n = 6). B, real-time PCR expression of F480 in livers of GRβ-Ad and vec-Ad mice. *, p < 0.05; **, p < 0.01 (versus vec-Ad mice) (± S.E.; n = 6).
Because high-fat feeding induces inflammation and possibly GC resistance (3), it is likely that increased expression of GRβ contributes to these phenotypes in response to an increase in high-fat intake. Although a high-fat diet increased GRβ levels in both adipose and liver, the levels of GRβ were not altered in muscle suggesting that skeletal muscle may not contribute to the first stages of high-fat diet-induced hepatic steatosis. Interestingly, the findings of He et al. (11) for the AAV-mediated delivery of human GRβ showed a reduced plasma glucose level, which is a conundrum because inflammation in the liver leads to glucose intolerance and high circulating insulin levels (42–45, 52–54). Furthermore, in the study by He et al. (11) hepatic lipid content was not measured, even though inflammation was present and is the precursor to fatty acid production in the liver (19, 34, 42–45). Our observation that overexpression of mGRβ caused an increase in M1 proinflammatory macrophage markers TNFα and iNOS as well as phosphorylation of NF-κB possibly by down-regulating IκBα, inhibition of GRα, or possibly through direct binding of GRβ-NF-κB heterodimers on promoters. NF-κB is a master transcription regulator of several genes involved in inflammation, and GRβ inhibition of GCs may play an important role. GRβ enhanced NF-κB activity at the NF-κB minimal and PTEN promoters. Progression from NAFLD to NASH can be accelerated via the loss of the tumor suppressor PTEN (56). GRβ overexpression resulted in a significant decrease in hepatic PTEN levels suggesting that down-regulation of this pathway may be another contributing factor to the development of hepatic steatosis in this model.

GRβ may serve to decrease the GRα-PPARα axis, which has been shown to be necessary for hepatic lipid catabolism (57). PPARα increases genes involved in the β-oxidation pathway, which cause the burning of fat. PPARα levels are significantly lower in the obese (19) and are increased during fasting and reduced with feeding (35). GRβ may be involved in the obesity-induced or feeding reduction of PPARα expression, as overexpression significantly decreased hepatic levels and we also showed it reduced transcriptional activity at the minimal PPRE
and FGF21 promoters. It is interesting to note that GRβ overexpression results in both enhanced GSK3β activity (decreased GSK3β phosphorylation) as well as lower levels of PPARα and its target gene, FGF21. PPARα also binds to the promoter of GYS2 to increase expression (58), which together interacts to regulate glycogen storage and steatosis. GSK3β is a negative regulator of GYS2 and decreases activity, causing alterations in hepatic glycogen production. We found reduced glycogen, but not significant, in the mice of this study, which were fasted and would have been producing glucose by glycogenolysis. Future studies on fed mice may show a significant reduction in glycogen during feeding with high GRβ levels, which is when glycogen is stored. GRβ overexpression resulted in a significant decrease in the levels of phosphorylation of serine 9 in GSK3β, which increases its activity. The decrease in GSK3β phosphorylation could be due to the lower Akt2 levels observed in the liver of GRβ overexpressing mice, as previous studies have demonstrated the regulation of GSK3β phosphorylation by the Akt pathway (59, 60).

GRβ overexpression resulted in a decrease in both the hepatic and serum levels of the PPARα target gene, FGF21. FGF21 is a hepatic hormone that has autocrine and exocrine effects that impact hepatic glycogen storage and reduces steatosis (Fig. 8) (12, 15, 16, 19, 38). It can also improve glycemic control and the management of body weight (13, 15–17, 19, 37–39, 61, 62). FGF21 expression is enhanced by both GRβ and the PPARα agonist WY-14,643 (WY). However, GRβ overexpression attenuated WY-14,643-induced FGF21 activation without any significant effect on basal FGF21 promoter activity. Mice lacking FGF21 have hepatic insulin resistance and increased glucose production from the liver (63), which may indicate that GRβ regulation of PPARα at the FGF21 promoter

**FIGURE 7.** GRβ inhibits PPARα transcriptional activity. A, transient transfection of GRβ, PPARα, RXRα, and PPARE-3tk-luc constructs in COS7 cells for 24 h following a 24-h WY-14,643 or vehicle (ctrl) treatment. ##, p < 0.01 (versus vector control); ###, p < 0.001 (versus vector WY) (± S.E.; n = 3). B, transient transfection of GRα, PPARα, RXRα, and PPARE-3tk-luc constructs in COS7 cells for 24 h following a 24-h WY-14,643 or vehicle (ctrl) treatment. ##, p < 0.01 (versus vector control); **, p < 0.001 (versus vector WY) (± S.E.; n = 3). C, transient transfection of GRβ, GRα, vector, and FGF21-luc constructs in COS7 cells for 24 h. **, p < 0.001 (versus vector) (± S.E.; n = 3). D, transient transfection of GRα, GRβ, PPARα, RXRα, and FGF21-luc constructs in COS7 cells for 24 h following a 24-h WY-14,643 or vehicle (ctrl) treatment. **, p < 0.01 (versus vector WY) (± S.E.; n = 3).
The experimental procedures and protocols of this study conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Toledo in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animal activity and grooming were monitored daily to assess overall animal health. Animals were housed in a temperature-controlled environment with 12-h dark-light cycle.

**Adenoviral Studies**

All mice were 8-week-old male C57BL/6 that were purchased from Jackson Labs. Mice were individually caged in specific pathogen-free enclosures with a 12-h light/dark cycle at 22 to 24 °C. All animals were fed a regular chow diet and injected with adenovirus as described below.

**Adenovirus Construction and Liver Overexpression**

To develop the adenovirus, mouse GRβ cDNA was inserted into BglII and XbaI restriction sites in the pAdTrack-CMV vector and the pAd-Easy system was used to build the virus as described in Ref. 66. The adenoviruses were multiplied in 293 HEK cells and purified using the AdEasy Virus Purification Kit (Agilent Technologies, catalogue number 240244-1). Either adenovirus overexpression GRβ (GRβ-Ad) or empty vector (vec-Ad) were injected by tail vein in mice. All tail vein injections were performed in a dedicated BSL2-certified animal holding room. Mice were anesthetized with inhaled isoflurane (1.5 to 2.5%) while on a warming pad. Although anesthetized, a warm pack was wrapped around the tail to cause vessel dilation. The tail was gently pulled down and a syringe was inserted into the tail vein. Animals remained in BSL2 housing for the duration of the study following injection (5 days). All adenovirus-infected mice were fed a regular diet chow (as described above) during the 5-day study. Mice were fasted overnight and then tissues and blood were harvested for analysis.

**Serum Analysis**

At the termination of the study, mice were fasted overnight and then euthanized. Blood was extracted from all animals for measurement of glucose, insulin, and FGF21. For glucose measurements ~3 to 5 μl were analyzed for blood glucose concentration using the laboratory animal-specific Alpha Trak glucometer and strips (Abbott Labs). The insulin concentrations were determined in serum samples of GRβ-Ad and vec-Ad mice using a commercially available ELISA as described by the provider (Crystal Chem) and as previously described (67). Serum triglycerides were determined with commercially available reagents (Pointe Scientific). The lower limit of detection for serum triglycerides was 5 mg/dl. FGF21 serum concentra-
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tions from mice fasted overnight were determined using an ELISA (Millipore). The lowest level of FGF21 detected by this test is 49.4 pg/ml. All samples were run in duplicate and diluted to fit within the respective standard curve. Samples were re-assayed if the coefficient of variance was greater than 10% for duplicates.

Liver Glycogen

Liver glycogen content was determined from fasted mice using a commercially available kit (Abnova). Briefly, 10 mg of tissue was homogenized in 200 µl of dH₂O using the Tissue Lyser bead homogenizer (Qiagen). Samples were boiled for 5 min and centrifuged at 13,000 rpm for 5 min. Then, 50-µl samples and standards were added to a 96-well plate and developed according to the manufacturer’s instructions. Samples were run in duplicate and samples were re-assayed if the coefficient of variance was greater than 10% or samples were outside the detectable limits of the test (0.0004 to 2 mg/ml), and multiplied by the appropriated dilution factor for actual concentrations.

Transfection and Promoter Luciferase Reporter Assays

Transient Transfection—Cells were plated on a 6-well dish in DMEM containing 10% calf serum prior to transfection and allowed to grow to 85–90% confluence. Cells were washed with Opti-MEM and transfected using GeneFect (Alkali Scientific, Inc.), according the manufacturer’s protocol. Opti-MEM was removed after 5 h and DMEM containing 10% FBS/serum was added.

Promoter Reporter Assays—Expression vector for mGRβ (pMGR-H57) was constructed as previously described (8). NF-κB minimal promoter-luciferase and PTEN promoter-luciferase activity was measured by firefly luciferase in the presence of the p65 subunit of NF-κB versus GRβ and/or GRα (PTEN-luc was made as defined in Ref. 6), and the pRL-CMV Renilla reporter for normalization to transfection efficiency. PPARα minimal promoter PPRE-3k-luciferase and FGF21-promoter-luciferase (gift from Dr. David Mangelsdorf at the University of Texas Southwestern) activity were measured by firefly luciferase in the presence of PPARα versus GRβ and/or GRα, and the pRL-CMV Renilla reporter for normalization to transfection efficiency. Transient transfection was achieved using GeneFect (Alkali Scientific, Inc.). Twenty-four-h post-transfected cells were treated for 24 h in dialyzed fatty acid-free FBS and then lysed. The luciferase assay was performed using the Promega dual luciferase assay system (Promega, Madison, WI).

Quantitative Real-time PCR Analysis

Total RNA was harvested from mice by lysing tissues using a Qiagen Tissue Lyser LT (Qiagen) and then extraction by a 5-Prime PerfectPure RNA Tissue Kit (Fisher Scientific Company, LLC). Total RNA was read on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR amplification of the cDNA was performed by quantitative real-time PCR using TrueAmp SYBR Green qPCR SuperMix (Alkali Scientific). The thermocycling protocol consisted of 5 min at 95 °C, 40 cycles of 15 s at 95 °C, and 30 s at 60 °C and finished with a melting curve ranging from 60 to 95 °C to allow distinction of specific products. Normalization was performed in separate reactions with primers to GAPDH mRNA.

Gel Electrophoresis and Western Blotting

Mouse tissues were flash frozen in liquid nitrogen during harvesting and stored at −80 °C. For gel electrophoresis, 50–100 mg of cut tissue was then resuspended in 3 volumes of CellLytic Buffer (Sigma C3228) plus 10% protease inhibitor mixture (Sigma P2714-1BTL) and Halt phosphatase inhibitor mixture (Fisher PI78420), and then, incubated on ice for 30 min followed by lysing the livers using a Qiagen Tissue Lyser LT (Qiagen) and then 100,000 × g centrifugation at 4 °C. Protein samples were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 2 h in TBS (10 mM Tris-HCl (pH 7.4) and 150 mM NaCl) containing 3% BSA. Subsequently, the membranes were incubated overnight at 4 °C with the following antibodies: PPARα (Santa Cruz Biotechnology, Santa Cruz, CA, sc-9000), heat shock protein 90 (HSP90) (Santa Cruz, 13119), Akt1 (Santa Cruz, sc-5298), Akt2 (Santa Cruz, sc-5270), fatty acid synthase (FAS) (Cell Signaling, 3180S), GSK3β (Cell Signaling, 9832S), phospho-GSK3β (Cell Signaling, 9336S), PTEN (Cascade Bioscience, ABM-2052), IκBα (Cell Signaling, 9242), NF-κB (Santa Cruz, sc-8008), phospho-S536 NF-κB (Cell Signaling, 3033), and FGF21 (Santa Cruz, sc-16842). After three washes in TBS + 0.1% Tween 20, the membrane was incubated with an infrared anti-rabbit (IRDye 800, green) or anti-mouse (IRDye 680, red) secondary antibody labeled with IRDye infrared dye (LI-COR Biosciences) (1:10,000 dilution in TBS) for 2 h at 4 °C. Immunoactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences).

Statistical Analysis

Data were analyzed with Prism 6 (GraphPad Software, San Diego, CA) using analysis of variance combined with Tukey’s post-test to compare pairs of group means or unpaired t tests. Results are expressed as mean ± S.E. Additionally, one-way analysis of variance with a least significant difference post hoc test was used to compare mean values between multiple groups, and a two-tailed, and a two-way analysis of variance was utilized in multiple comparisons. p values of 0.05 or smaller were considered statistically significant.

Author Contributions—T. D. H. and J. S. M. conceived and coordinated the study. J. S. M., L. A. S., D. E. S., and T. D. H. wrote the manuscript. J. S. M., L. A. S., and T. D. H. designed, performed, and analyzed the experiments shown in Figs. 1–7, and contributed to the preparation of the figures. T. D. H. and J. S. M. designed and generated the GRβ adenovirus with guidance from D. E. S. A. N. K. performed all imaging in the manuscript. S. C. and L. A. S. performed real-time PCR analysis. All authors reviewed the results and approved the final version of the manuscript.

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