Histone Deacetylase 6 (*HDAC6*) Is an Essential Modifier of Glucocorticoid-Induced Hepatic Gluconeogenesis

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In the current study, we investigated the importance of histone deacetylase (HDAC)6 for glucocorticoid receptor–mediated effects on glucose metabolism and its potential as a therapeutic target for the prevention of glucocorticoid-induced diabetes. Dexamethasone-induced hepatic glucose output and glucocorticoid receptor translocation were analyzed in wild-type (wt) and HDAC6-deficient (HDAC6KO) mice. The effect of the specific HDAC6 inhibitor tubacin was analyzed in vitro. wt and HDAC6KO mice were subjected to 3 weeks' dexamethasone treatment before analysis of glucose and insulin tolerance. HDAC6KO mice showed impaired dexamethasone-induced hepatic glucocorticoid receptor translocation. Accordingly, dexamethasone-induced expression of a large number of hepatic genes was significantly attenuated in mice lacking HDAC6 and by tubacin in vitro. Glucose output of primary hepatocytes from HDAC6KO mice was diminished. A significant improvement of dexamethasone-induced whole-body glucose intolerance as well as insulin resistance in HDAC6KO mice compared with wt littermates was observed. This study demonstrates that HDAC6 is an essential regulator of hepatic glucocorticoid-stimulated gluconeogenesis and impairment of whole-body glucose metabolism through modification of glucocorticoid receptor nuclear translocation. Selective pharmacological inhibition of HDAC6 may provide a future therapeutic option against the prodiabetogenic actions of glucocorticoids. ***Diabetes*** **61:513–523, 2012**

Glucocorticoids exert potent anti-inflammatory actions and are widely used in patients suffering from rheumatoid arthritis, asthma, and skin disorders or after organ transplantation (1). On the other hand, the use of glucocorticoids, especially when chronically administered in high doses, is still limited by serious side effects. Patients treated with glucocorticoids have a markedly increased risk for developing hypertension, osteoporosis, or hyperglycemia as a result of augmented gluconeogenesis and other anti-insulinemic effects. Up to 25% of all patients treated with high-dose glucocorticoids even develop persistent steroid diabetes, which thus far requires discontinuation of glucocorticoid therapy or antidiabetes treatment (2–7). Therefore, new options for therapeutic intervention are directly needed to improve the drug profile of glucocorticoids.

On the molecular level, glucocorticoids readily diffuse through the cell membrane because of their lipophilic nature and exert their effects by binding to the glucocorticoid receptor. The glucocorticoid receptor, also known as NR3C1, belongs to the family of nuclear receptors and binds cortisol as endogenous ligand in humans as well as exogenous, synthetic glucocorticoids such as dexamethasone. Upon ligand binding, the receptor-ligand complex translocates to the nucleus, where it serves as a transcriptional regulator by either binding to specific glucocorticoid-responsive elements (GREs), which can be positive or negative GREs, or by interacting with other proteins that function as transcriptional regulators (8–11).

In absence of ligand, the glucocorticoid receptor resides primarily in the cytoplasm in a complex with the chaperones heat shock protein (HSP)90 and HSP70 and the cochaperones HSP70-HSP90 organizing protein, Hsp40, p23, and others (12). Glucocorticoid receptor–chaperone complex formation is required in order to achieve a competent glucocorticoid receptor conformation for high-activity ligand-binding ability. Disruption of the HSP90–glucocorticoid receptor complex is associated with loss of dexamethasone-binding activity, reduced translocation of the glucocorticoid receptor–glucocorticoid complex into the nucleus, and eventually inhibited glucocorticoid receptor–mediated transcriptional effects (10,13–16).

Several studies have demonstrated that this disruption can be achieved by compounds such as geldanamycin, an HSP90 inhibitor, but also by altering the acetylation status of HSP90, which determines ATP-binding ability and therefore the binding ability of HSP90 to its client proteins and cochaperones (13,17–20). In this context, much attention has been brought to the histone deacetylase (HDAC)6, which is known to deacetylate several nonhistone proteins such as HSP90, cortactin, tubulin, β-catenin, and peroxiredoxin (17,21–25). Since HDAC6 contains intrinsic nuclear export signals, it is almost exclusively located in the cytoplasm and therefore regarded as not having any transcription-modulating effects due to histone deacetylation (26,27). Inhibition of HDAC6 activity results in hyperacetylation of HSP90 and prevents its deacetylation. Since deacetylation of HSP90, in particular at the Lys294 residue, is a prerequisite for glucocorticoid receptor–HSP90 complex assembly, blockade or ablation of HDAC6 has been shown to lead to impaired chaperone-dependent activation of the glucocorticoid receptor (22,28,29).

Compounds that inhibit HDAC activity have been developed rapidly over the last years as anticancer drugs, and some, like vorinostat or romidepsin, have already been

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approved for clinical use. The benefit-risk profile of these HDAC inhibitors is expected to ameliorate with rising selectivity. We included tubacin, a first-in-class HDAC6-selective inhibitor (30), in our in vitro experiments to test whether pharmacological blockade of HDAC6 can modify glucocorticoid-mediated metabolic actions.

In the current study, we identified HDAC6 as a potential target for ameliorating glucocorticoid-induced hyperglycemia, glucose intolerance, and insulin resistance at least in part by impairing glucocorticoid receptor–mediated hepatic induction of gluconeogenesis. This results from a reduced translocation of the glucocorticoid receptor into the nucleus and subsequent diminished glucocorticoid receptor–induced transcription of key gluconeogenic genes. Accordingly, we were able to show effects on glucocorticoid receptor–mediated transcription by selective pharmacological HDAC6 inhibition by tubacin.

RESEARCH DESIGN AND METHODS

Male HDAC6-deficient (HDAC6KO) mice on a C57Bl/6 background and their wild-type (wt) littermates were generously supplied by P.M. (29). Eight-week-old mice were randomly assigned to vehicle or dexamethasone treatment for 3 weeks (n = 4–9/group). Dexamethasone (1 mg/kg) and vehicle (0.9% NaCl) were given every other day via intraperitoneal injection. Body weight was determined throughout the experiment. After 3 weeks of treatment, a glucose tolerance test (GTT) was carried out after fasting the mice overnight, injecting glucose intraperitoneally, and measuring the glucose concentration in peripheral blood taken from the tail vein at time points 0, 30, 60, 90, and 120 min. At the end of the study, animals were killed and organs shock frosted in liquid nitrogen for subsequent isolation of RNA and proteins. Serum insulin levels were determined by ELISA according to the manufacturer's instructions (Linco Research). The insulin tolerance test (ITT) was performed on a second set of animals (n = 5 per group) that were also 8 weeks of age at the beginning of the treatment. These animals were injected with 1 mg/kg/day dexamethasone and vehicle, respectively, for 3 weeks before the ITT was conducted. For the experiment, the animals were injected with insulin (0.5 units/kg body wt i.p.) after a 4-h fasting period. The glucose concentration in peripheral blood taken from the tail vein was measured at time points 0, 15, 30, 60, 90, 120, 150, and 180 min postinjection. At the end of treatment, serum corticosterone levels were assessed by radioimmunoassay. All animal procedures were in accordance with institutional guidelines and were approved.

Primary hepatocyte isolation. HDAC6KO and wt animals were anesthetized, and livers were perfused with perfusion and collagenase digestion buffer via the vena cava. Cells were separated by spinning in Percoll buffer, washed, eventually counted, checked for viability, and plated on collagen I–coated plates. After 4 h, medium was renewed. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% FBS and 1% penicillin/streptomycin (all from GIBCO). For ex vivo stimulation of primary hepatocytes, cells were starved for 2 h before pretreatment with 5 μmol/L tubacin (Enzo Life Sciences) for 2 h, with subsequent stimulation with dexamethasone (500 nmol/L for 6 h).

Glucose output. For analysis of hepatic glucose output, primary hepatocytes were serum starved overnight and then injected with vehicle, dexamethasone (500 nmol/L), or glucagon (100 nmol/L)/ cAMP (1 μmol/L). Twenty hours after treatment, medium was changed to glucose-free medium supplemented with sodium pyruvate (2 mmol/L) and sodium lactate (20 mmol/L) and dexamethasone (500 nmol/L), glucagon (100 nmol/L), and cAMP (1 μmol/L) or vehicle for 4 h. Eventually, 500 μL culture medium was taken to measure glucose concentration (Amplex Red Glucose Assay kit; Invitrogen). Glucose content was normalized to total protein amount.

Ex vivo lipolysis assay. Lipolysis from gonadal fat pads was analyzed as previously described (31). Stimulation with compounds is described in Fig. 5.

Cell culture and quantitative real-time PCR. The H4IIE rat hepatoma cells and human acute monocytic leukemia THP-1 cells were purchased from American Type Culture Collection, cultured according to the manufacturer's instructions, and stimulated as indicated in Fig. 5*C*. Quantitative real-time PCR was performed as previously described (31). Primer sequences are available on request.

Western blotting. Western blotting was performed as previously described (31). The following primary/secondary antibodies were used: glucocorticoid receptor (1:500; Santa Cruz Biotechnology), α-tubulin (1:1,000; Sigma), histone H4 (cat. no. 2935, 1:1,000; Cell Signaling Technology), HDAC6 (1:500; Acris Antibodies), and peroxidize-conjugated (1:2,000; Dako) antibodies. Fractionation of cellular proteins into nuclear and cytoplasmic parts was performed by grinding an exact amount of liver tissue in a Dounce homogenizer using buffer A (50 mmol/L Tris, pH 7.2; 250 mmol/L sucrose; 10 mmol/L KCl; 1 mmol/L EDTA; 10 mmol/L NaF; 2 mmol/L Na_3VO_4; 1 mmol/L dithiothreitol; and 1 mmol/L phenylmethylsulfonyl fluoride). This suspension was centrifuged and the supernatant frozen as cytoplasmic fraction. The pellet was resuspended in buffer B (50 mmol/L Tris, pH 7.2; 25% glycerol; 420 mmol/L NaCl; 1.5 mmol/L $MgCl_2$; 10 mmol/L NaF; 1 mmol/L dithiothreitol; 1 mmol/L phenylmethylsulfonyl fluoride, 0.1% SDS, and 1% Nonidet P-40) and rotated on a wheel for 30 min at 4°C. After centrifugation, supernatant was taken as nuclear enriched fraction.

Microarray analysis. Total RNA was prepared from livers of 12 mice (n = 3 for each group) and used for a whole-genome microarray (Mouse Gene 1.0; Affymetrix) as previously described (32). Complete datasets are available in the Gene Expression Omnibus database.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed using the Low Cell# ChIP kit (Diagenode) according to the manufacturer's instructions. First antibodies to the proteins of interest (acetyl-H3K9 [Millipore], H3 total [Cell Signaling Technology], acetyl-H4K8 [Cell Signaling Technology], H4 total [Cell Signaling Technology], RNA-polymerase II [Millipore], and IgG [Diagenode]) were bound to magnetic beads. Chromatin was cross-linked for 15 min at 37°C using 1% formaldehyde and washed twice with ice-cold phosphate-buffered saline. The cross-linking reaction was stopped by incubation with Gly (0.125 mol/L) for 5 min. Chromatin was sheared by controlled sonification using the Bioruptor (Diagenode), incubated with antibody-coated beads overnight. Chromatin immunoprecipitation DNA fragments were purified, and DNA sequences were determined by quantitative PCR. Primer sequences are available on request. Standardization was the recruitment of RNA polymerase II to the human TATA box region of the glyceraldehyde-3-phosphate dehydrogenase promoter; input served as positive control and IgG as negative control. Further negative controls were primer pairs outside of the *PEPCK* promoter; with an intergenic primer pair (proximal site), that means within *PEPCK* promoter sequence but outside the GRUs. Results represent at least data from five independent experiments.

Statistical analysis. Differences between the groups were assessed by ANOVA with the least significant difference/Bonferroni correction posttest, except where indicated. The results are expressed as means ± SEM, and $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the GraphPad software.

RESULTS

HDAC6 deficiency inhibits hepatic ligand-dependent glucocorticoid receptor nuclear translocation. HDAC6 exerts an important function toward dexamethasone-binding activity of the glucocorticoid receptor. As we have previously shown in mouse embryonic fibroblasts from HDAC6KO mice, HSP90 is hyperacetylated and glucocorticoid receptor translocation and transcriptional activity are reduced upon ligand binding (29). To assess whether there is a similar regulation in liver, hepatic glucocorticoid receptor translocation was analyzed in chronically glucocorticoid- or vehicle-injected HDAC6KO mice and wt littermates. After administration for 3 weeks of dexamethasone (1 mg/kg body wt) or vehicle, animals were killed and livers excised 3 h after the last vehicle/dexamethasone injection. Part of the liver was processed to provide nuclear and cytoplasmic protein fractions. Analysis of protein expression in these subcellular fractions revealed translocation of the glucocorticoid receptor from cytoplasm to the nucleus after glucocorticoid treatment in wt animals. Glucocorticoid-mediated glucocorticoid receptor translocation was markedly diminished in HDAC6KO mice. α-Tubulin and histone H4 were used as cytoplasmic and nuclear control proteins (Fig. 1*A* and *B*). These data demonstrate that in vivo hepatic glucocorticoid-induced nuclear translocation of the glucocorticoid receptor is impaired in the absence of HDAC6.

Influence of HDAC6 on hepatic gene expression. To examine whether impaired glucocorticoid receptor translocation in HDAC6KO mice translates into changes of glucocorticoid receptor–mediated hepatic gene regulation, gene expression analysis in livers from HDAC6KO or wt littermates was performed using DNA microarrays (Affymetrix).

By comparing samples from wt and HDAC6KO mice, we found a large number of genes being regulated by dexamethasone treatment in both groups. However, in a large set of genes, dexamethasone-mediated regulation was attenuated in livers from HDAC6KO mice compared with those in wt mice (Table 1). To see in more detail whether known glucocorticoid receptor target genes are differently regulated in both mouse strains, we picked *DUSP1* and *SGK1* for a confirmatory expression analysis by quantitative real-time PCR. The results showed that dexamethasone-induced gene expression was significantly attenuated in livers from HDAC6KO mice compared with those from wt littermates (Fig. 2*A* and *B*). Taken together, these results indicate that inhibition of glucocorticoid receptor translocation in HDAC6KO livers results in attenuated glucocorticoid receptor–mediated gene expression, identifying HDAC6 as a modulator of hepatic glucocorticoid receptor actions.

HDAC6 affects glucocorticoid-induced hepatic glucose metabolism. We next analyzed the expression of glucocorticoid receptor target genes involved in gluconeogenesis as an important metabolic pathway in the development of glucocorticoid-induced diabetes. We compared the gene expression of the key gluconeogenic enzymes glucose 6-phosphatase (*G6P*), fructose 1,6-bisphosphatase (*FBP*), *PEPCK*, and pyruvate carboxylase (*PCX*) in liver samples from HDAC6KO mice and wt littermates. All four genes were significantly upregulated in dexamethasone-treated wt mice (Fig. 3*A–D*). In HDAC6KO mice, dexamethasone-induced gluconeogenic gene expression in liver was significantly attenuated compared with that in wt littermates. The most prominent diminution of dexamethasone-mediated stimulation was detected for *G6P* and *FBP*, whereas induction of *PEPCK* and *PCX* was only moderately but significantly attenuated (Fig. 3*A–D*). To expand these results, we used an additional approach to block HDAC6 activity by pharmacological inhibition. H4IIE rat hepatoma cells (Fig. 3*E* and *F*) and primary murine hepatocytes (Fig. 3*G* and *H*) were stimulated with the HDAC6-selective inhibitor tubacin or the pan-HDAC inhibitor trichostatin A prior to stimulation with dexamethasone. We analyzed *G6P* as well as *PEPCK* gene expression after 4 h (H4IIE) and 6 h (primary hepatocytes) of dexamethasone stimulation and demonstrated that pharmacological inhibition of HDAC6 also abrogates dexamethasone-induced *G6P* and *PEPCK* gene expression in liver cells (Fig. 3*E–H*).

To assess whether this attenuation of glucocorticoid receptor–regulated gene expression by HDAC6 deficiency is adequate for affecting hepatic gluconeogenesis, we investigated glucose production of primary hepatocytes. Hepatocytes from HDAC6KO mice and wt littermates were serum starved overnight before stimulation of hepatic gluconeogenesis with dexamethasone. Glucose output was determined as described in the RESEARCH DESIGN AND METHODS. After dexamethasone stimulation, we observed a significant increase in glucose production in wt hepatocytes (Fig. 3*I*). This increase was significantly reduced in HDAC6KO hepatocytes (Fig. 3*I*). In contrast, glucose output induced by glucagon and cAMP was not affected by HDAC6 deficiency (Fig. 3*I*). In summary, loss of HDAC6 activity results in attenuation of hepatic glucocorticoid receptor–induced gluconeogenic gene expression, diminishing glucocorticoid-mediated glucose production.

HDAC6 regulates hepatic histone acetylation. Previous reports have shown that HDAC6, in addition to its cytoplasmatic actions, directly regulates gene transcription in the nucleus via interaction with regulatory promoter regions (34,35). To assess whether HDAC6 plays a role in histone deacetylation on gluconeogenic gene promoters

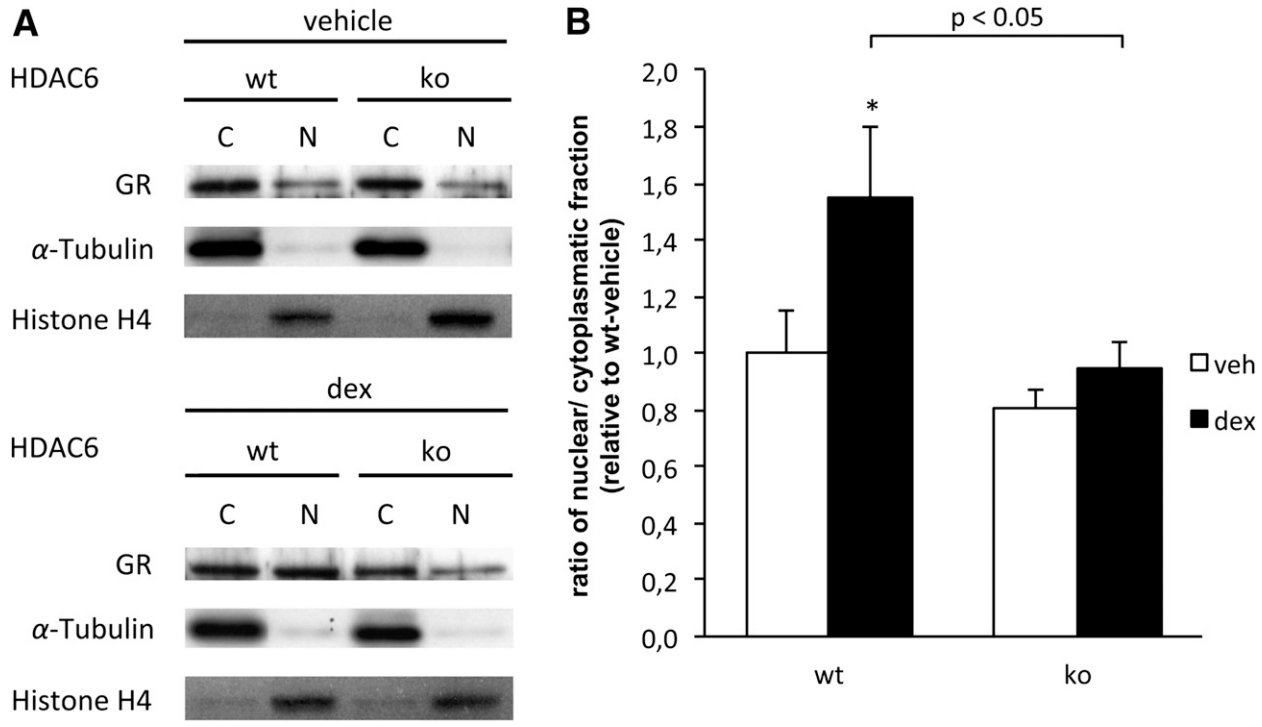

FIG. 1. Impaired glucocorticoid-induced glucocorticoid receptor (GR) translocation attributed to lack of HDAC6. *A*: Nuclear (N) and cytoplasmic (C) protein fractions of livers from wt and HDAC6KO (ko) mice were Western blotted for glucocorticoid receptor. Representative immunoblots from three sets of animals are shown. *B*: Densitometric analysis from HDAC6 Western immunoblots as the ratio of nuclear to cytoplasmatic fraction. (See *A*.) *$P < 0.05$ vs. wt plus vehicle (veh). dex, dexamethasone.

TABLE 1
Microarray analysis of hepatic mRNA

x-fold induction by dexamethasone in wt mice	x-fold induction by dexamethasone in HDAC6KO mice	Gene symbol	Description
13.26	6.76	*Dusp1*	Dual specificity phosphatase 1
19.11	9.79	*Acot1*	Acyl-CoA thioesterase 1
−8.11	−4.27	*Gatm*	Gly amidinotransferase
13.60	7.43	*Treh*	Trehalase (brush-border membrane glycoprotein)
−8.12	−4.48	*Gnpda1*	Glucosamine-6-phosphate deaminase 1
8.22	4.54	*Slc25a33*	Solute carrier family 25, member 33
9.98	5.55	*Accn5*	Amiloride-sensitive cation channel 5, intestinal
−7.83	−4.36	*Sirpa*	Signal-regulatory protein α
−8.94	−5.01	*Tmie*	Transmembrane inner ear
8.75	4.95	*Porcn*	Porcupine homolog (Drosophila)
−11.19	−6.46	*Fnip2*	Folliculin interacting protein 2
−12.31	−7.19	*Trim14*	Tripartite motif-containing 14
8.45	5.02	*Skil*	SKI-like
−9.61	−5.79	*Dclk3*	Doublecortin-like kinase 3
−8.53	−5.26	*Gpr146*	G-protein–coupled receptor 146
−8.84	−5.46	*Anks4b*	Ankyrin repeat and sterile α motif domain containing 4B
−9.64	−5.97	*Lima1*	LIM domain and actin-binding 1
7.04	4.36	*5730446C15Rik*	RIKEN cDNA 5730446C15 gene
−7.58	−4.80	*Pola2*	Polymerase (DNA directed), α 2
−9.56	−6.13	*D430015B01Rik*	RIKEN cDNA D430015B01 gene
−6.70	−4.29	*Plekhg6*	Pleckstrin homology domain containing, family G member 6
−7.20	−4.62	*Notch2*	Notch gene homolog 2 (Drosophila)
8.05	5.20	*Slc25a25*	Solute carrier family 25, member 25
−6.67	−4.32	*ENSMUSG00000057924*	Predicted gene, ENSMUSG00000057924
−7.84	−5.12	*Es22*	Esterase 22
−6.63	−4.34	*Centd2*	Centaurin, delta 2
−7.53	−4.94	*Arrdc4*	Arrestin domain containing 4
−6.87	−4.53	*Smad3*	MAD homolog 3 (Drosophila)
−8.22	−5.50	*Irf1*	Interferon regulatory factor 1
−7.49	−5.02	*Sept9*	Septin 9
−9.43	−6.38	*Svil*	Supervillin
−7.45	−5.05	*Oaf*	OAF homolog (Drosophila)
11.68	7.94	*4930485B16Rik*	RIKEN cDNA 4930485B16 gene
−8.58	−5.90	*Olfml1*	Olfactomedin-like 1
−6.79	−4.67	*EG545242*	Predicted gene, EG545242
8.31	5.74	*Gadd45 g*	Growth arrest and DNA-damage-inducible 45 γ
6.94	4.84	*Sept14*	Septin 14
−7.46	−5.21	*Cybasc3*	Cytochrome b, ascorbate dependent 3
−9.55	−6.68	*1810031K17Rik*	RIKEN cDNA 1810031K17 gene
−10.63	−7.50	*Plxnd1*	Plexin D1

Microarray analysis of hepatic mRNA was performed. Displayed are the strongest x-fold inductions/inhibitions of dexamethasone-treated wt mice compared with vehicle-injected wt mice and HDAC6KO mice, respectively (n = 3).

in the liver, we performed chromatin immunoprecipitation experiments from livers of wt and HDAC6KO mice (Fig. 4*A*–*D*) and investigated acetylation marks of substrate histones associated with the *PEPCK* gene. The degree of acetylation of Lys9 on histone H3 (H3K9) and of Lys8 on histone H4 (H4K8) was analyzed. Absence of HDAC6 resulted in slightly augmented acetylation of histone H3 and H4; only the acetylation of H4K8 reached statistical significance (Fig. 4*A*–*D*). Since acetylation of histone H3 and H4 is closely associated with active transcription (36), these data may suggest that the loss of HDAC6 in the liver results in an attenuation of glucocorticoid-induced gene expression via suppression of glucocorticoid receptor translocation, which may be counterregulated by parallel hyperacetylation of histones associated with the target promoter and activation of transcription.

Regulation of glucocorticoid-mediated actions by HDAC6 in adipose tissue and monocytes. To determine the importance of HDAC6 for glucocorticoid/glucocorticoid receptor–mediated effects in other tissues/cells, we first studied the expression level of HDAC6 in liver, skeletal muscle, and gonadal adipose tissue. As depicted in Fig. 5*A*, highest expression of HDAC6 protein was detected in liver, followed by skeletal muscle and adipose tissue.

To investigate whether HDAC6 deficiency also impairs glucocorticoid receptor function in adipose tissue, ex vivo lipolysis was measured in gonadal adipose tissue from wt and HDAC6KO mice (Fig. 5*B*). As previously reported, dexamethasone stimulation resulted in a significant induction of basal adipose tissue lipolysis in wt mice (Fig. 5*B*). In adipose tissue from HDAC6KO mice, dexamethasone-induced lipolysis was attenuated but still present compared with that in untreated controls. In addition, differences

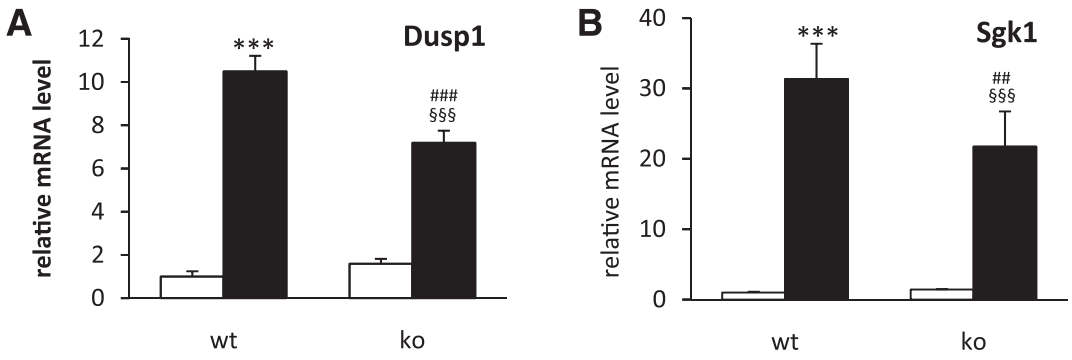

FIG. 2. Attenuation of dexamethasone-induced hepatic mRNA regulation in HDAC6KO (ko) mice (*A*) and real-time quantitative PCR analysis of *Dusp1* and *Sgk1* showing attenuated dexamethasone-induced gene regulation in HDAC6KO mice (*B*). Data represent means ± SEM (n = 7–8). ***P < 0.001 vs. wt plus vehicle, ###P < 0.001 vs. HDAC6KO plus vehicle, §§§P < 0.001 vs. wt plus vehicle. ■, dexamethasone; □, vehicle.

between dexamethasone-stimulated lipolysis were not statistically significant comparing wt and HDAC6KO mice, suggesting that the relevance of HDAC6 for glucocorticoid action may differ in adipose tissue compared with the liver.

We also determined the role of HDAC6 in glucocorticoid-mediated anti-inflammatory actions. Human THP-1 monocytes were treated with lipopolysaccharide (LPS), with or without dexamethasone, and the selective HDAC6 inhibitor tubacin, and mRNA expression of interleukin (IL)-6 was analyzed. As shown in Fig. 5*C*, LPS stimulation resulted in a pronounced induction of interleukin-6 (*IL-6*) mRNA expression, which was potently inhibited by dexamethasone. In the presence of selective HDAC6 inhibition by tubacin, dexamethasone was still able to suppress LPS-induced *IL-6* induction, suggesting a minor regulatory function for HDAC6 in monocytic glucocorticoid/glucocorticoid receptor effects.

HDAC6 deficiency ameliorates glucocorticoid-induced diabetogenic effects. Does HDAC6 inhibition possess the potential for improving glucocorticoid-induced insulin resistance and glucose intolerance? To address this question, we injected wt and HDAC6KO mice with dexamethasone (1 mg/kg body wt) or vehicle for 3 weeks and performed GTTs and ITTs.

At the end of the treatment period, absolute body weights did not significantly differ between the groups (wt plus vehicle 26.9 ± 0.7 g, wt plus dexamethasone 24.9 ± 0.8 g, HDAC6KO plus vehicle 27.2 ± 0.6 g, and HDAC6KO plus dexamethasone 25.0 ± 0.8 g). Mean food intake during 24 h (analyzed at the end of the treatment period) was similar between the groups (measured in grams per 24 h): wt plus vehicle 3.6 ± 0.4, wt plus dexamethasone 3.7 ± 0.2, HDAC6KO plus vehicle 3.8 ± 0.2, and HDAC6KO plus dexamethasone 3.3 ± 0.3. Glucocorticoid-treated wt animals exhibited a mild fasting hyperglycemia that was absent in all other groups (Fig. 6*A*). Furthermore, in wt mice dexamethasone treatment resulted in an impairment of glucose tolerance shown in the glucose tolerance curve (Fig. 6*B*) and the calculated relative area under the curve (AUC) for glucose concentration (Fig. 6*C*). Strikingly, when looking at the HDAC6KO counterparts we did not observe any significant changes in fasting glucose or glucose tolerance after dexamethasone administration (Fig. 6*A*–*C*). To exclude the possibility of shortened pancreatic insulin supply and subsequent impairment of glucose utilization, we determined serum insulin levels of the animals by enzyme-linked immunosorbent assay. We observed an almost fivefold increase of serum insulin concentration in wt mice after dexamethasone treatment (Fig. 6*D*). In contrast, glucocorticoid administration did not affect serum insulin levels in HDAC6KO animals (Fig. 6*D*). These data point toward a glucocorticoid-mediated insulin resistance associated with hyperinsulinemia, which is alleviated in HDAC6KO mice. To verify this conclusion, we conducted an ITT, which disclosed a strikingly impaired response to insulin in dexamethasone-treated wt mice (Fig. 6*E* and *F*). In HDAC6KO animals, dexamethasone-mediated insulin resistance was markedly attenuated compared with that in wt mice (Fig. 6*E* and *F*).

Finally, we performed biochemical analysis of the hypothalamic-pituitary-adrenal axis by measuring endogenous glucocorticoids (corticosterone) in wt and HDAC6KO mice that did or did not receive dexamethasone treatment. Serum corticosterone levels were 60 ± 7 ng/mL in untreated wt and 86 ± 16 ng/mL in untreated HDAC6KO mice. Dexamethasone treatment markedly suppressed corticosterone levels in both genotypes below detection level (<7.7 ng/mL, radioimmunoassay), suggesting the presence of an intact hypothalamic-pituitary-adrenal axis in wt and HDAC6KO mice.

The results taken together demonstrate that HDAC6 deficiency results, at least in part, in prevention of glucocorticoid-induced hyperglycemia, glucose intolerance, and insulin resistance.

DISCUSSION

The current study demonstrates that HDAC6 is important for hepatic glucocorticoid receptor nuclear translocation and required for a full glucocorticoid-mediated response in relation to hepatic gene expression. Attenuation of glucocorticoid receptor translocation through loss of HDAC6 activity results in diminution of glucocorticoid-induced gluconeogenic gene expression followed by decreased rates of hepatic glucose production. Accordingly, HDAC6 deficiency abrogates prodiabetogenic actions of glucocorticoids, including prevention of fasting hyperglycemia, glucose intolerance, and insulin resistance.

Previously, HDAC6 was shown to influence glucocorticoid receptor–HSP90 heterocomplex assembly and subsequent translocation of the glucocorticoid receptor into the nucleus in various cell systems such as mouse embryonic fibroblasts or A549 epithelial cells (22,29). In the current study, we demonstrate that loss of HDAC6 activity also impairs glucocorticoid-induced glucocorticoid receptor translocation from cytoplasm to nucleus in vivo, in particular in

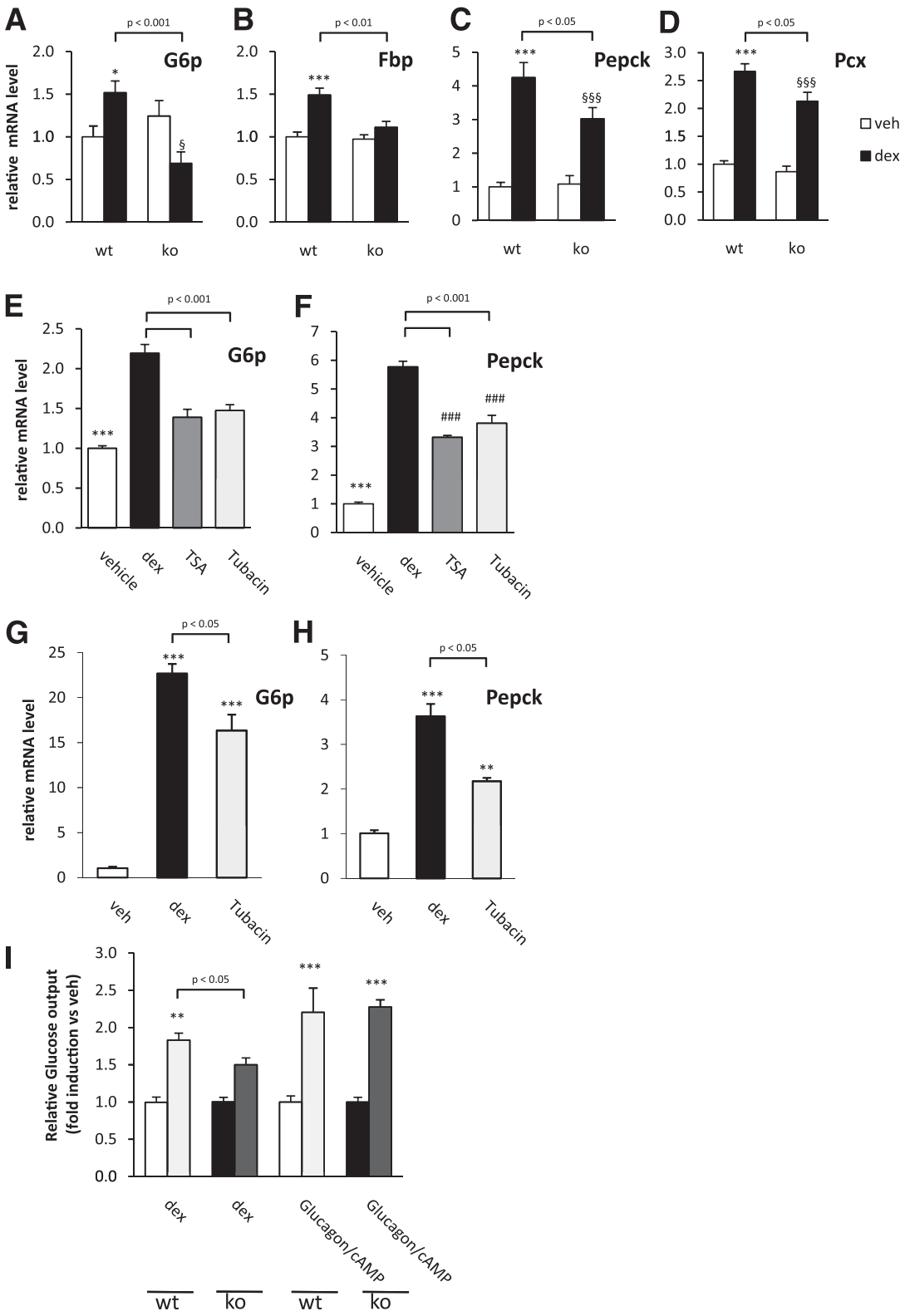

FIG. 3. Regulation of gluconeogenic genes by dexamethasone (dex) in the liver is attenuated in HDAC6KO (ko) mice. *A–D*: Real-time quantitative PCR analysis of *G6p*, *Fbp*, *Pepck*, and *Pcx* of animal liver mRNA (n = 7–8 mice). *E* and *F*: H4IIE cells were stimulated with dexamethasone (500 nmol/L) for 4 h after 30-min pretreatment with vehicle (veh) and HDAC inhibitors (5 μmol/L tubacin and 1 μmol/L trichostatin A [TSA]). Real-time quantitative PCR analysis was conducted for the respective genes. *G* and *H*: Primary murine hepatocytes were stimulated with dexamethasone (500 nmol/L) after pretreatment with vehicle or tubacin (5 μmol/L). Real-time quantitative PCR analysis was conducted for the respective genes. Data represent means ± SEM of three independently conducted experiments. *I*: wt and HDAC6KO primary hepatocytes were treated with dexamethasone (10 nmol/L) and glucagon (100 nmol/L)/cAMP (1 μmol/L). Glucose content of the supernatant was quantified and normalized to total protein amount. Glucose output was conducted three times in triplicates. Data represent means ± SEM. *A–D*: *P < 0.05, ***P < 0.001 vs. wt plus vehicle; §P < 0.05, §§§P < 0.001 vs. HDAC6KO plus vehicle. *E–F*: ***P < 0.001, ###P < 0.001 vs. vehicle. *G–I*: ***P < 0.001, **P < 0.01 vs. respective vehicle.

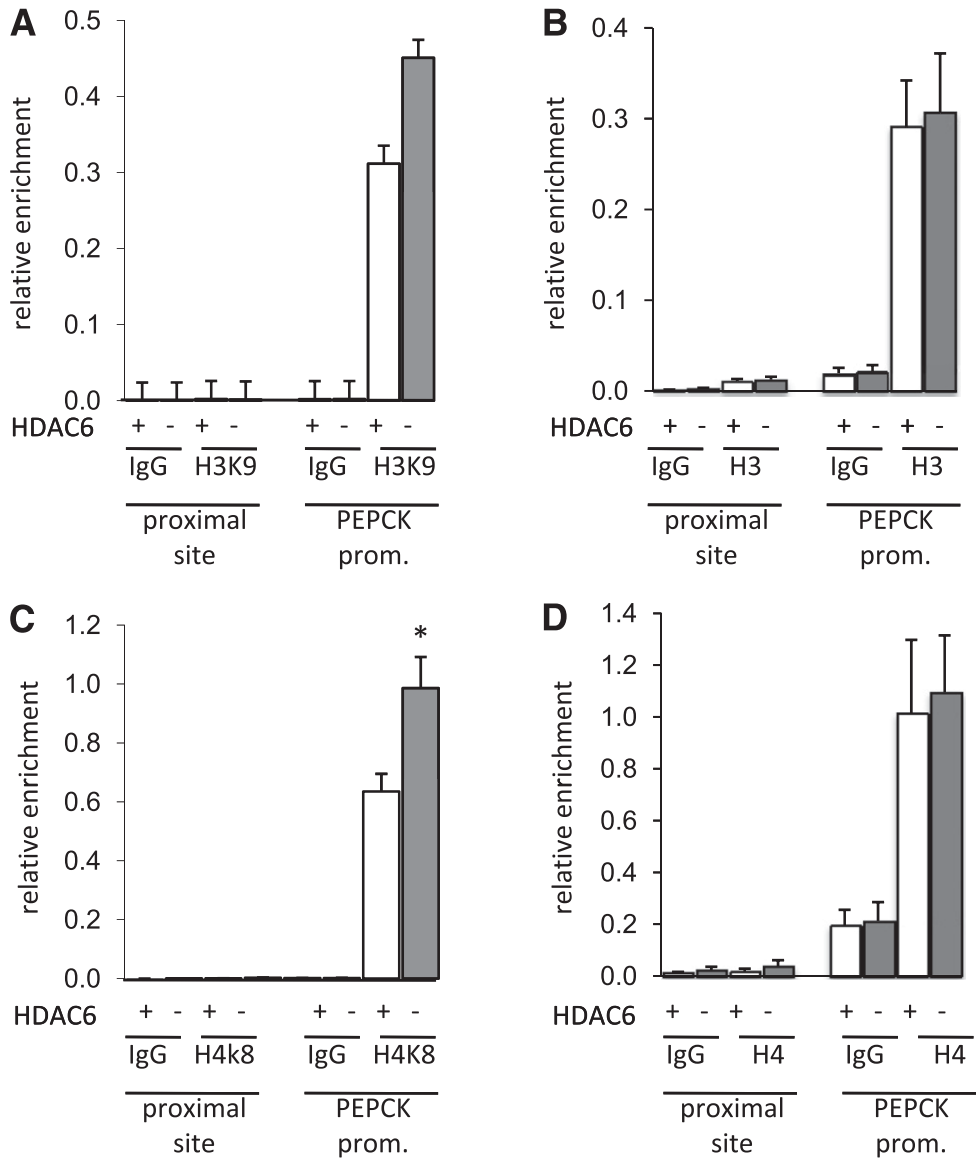

FIG. 4. HDAC6 regulates hepatic histone acetylation. Chromatin immunoprecipitation (ChIP) analysis of the *PEPCK* promoter (prom.) shows histone H3 and H4 acetylation in livers from wt (+) and HDAC6KO (−) mice. Details can be found in RESEARCH DESIGN AND METHODS. The amount of acetylated H3K9 (*A*) and H4K8 (*C*) (proximal site: IgG, H4K8. *PEPCK* promoter [prom.]: IgG, no detectable signal after adequate amplification) and total H3 (*B*) and H4 (*D*) associated with the *PEPCK* promoter was analyzed. Results represent data from three independent experiments. *$P < 0.05$ vs. wt.

liver, the organ of major metabolic glucocorticoid actions. However, translocation of the glucocorticoid receptor was not completely abrogated in HDAC6KO livers/hepatocytes. This is in accordance with Murphy et al. (13) showing that even in HDAC6 knockdown cells, some HSP90 still remains with high ATP-binding affinity and therefore keeps the ability to form HSP90–glucocorticoid receptor complexes. In our study, this resulted in attenuated glucocorticoid response in relation to hepatic gene expression and not in a complete blockade of glucocorticoid action. It should be emphasized that the present model substantially differs from other models in which glucocorticoid/GRE-dependent actions are inhibited more completely such as in GR^{dim} mice. In these mice, a point mutation impairs glucocorticoid receptor dimerization and almost completely blocks glucocorticoid/glucocorticoid receptor–dependent transactivation (37,38). Furthermore, attenuation of glucocorticoid-induced gene expression by HDAC6 deficiency differed quantitatively depending on the individual target gene. This observation is in accordance with a gene-specific sensitivity to dexamethasone stimulation in liver as shown in our study (Fig. 3*A*–*D*) and in previous studies (33). The underlying molecular mechanism likely involves promoter-specific composition of regulatory GRUs. As described for the *PEPCK* and *G6P* promoter, GRUs consist of an array of binding sites for so-called accessory transcription factor located in proximity to the glucocorticoid receptor binding sites (39). Coordinated binding of these factors including hepatocyte nuclear factor-4, retinoid X receptor α, and others to the target promoter together with ligand-activated glucocorticoid receptor is required for a full pharmacological response to glucocorticoids (40,41). Thus, distinct HDAC6-mediated modification of glucocorticoid-induced gluconeogenic gene expression is likely explained by promoter-specific sensitivity to glucocorticoids based on

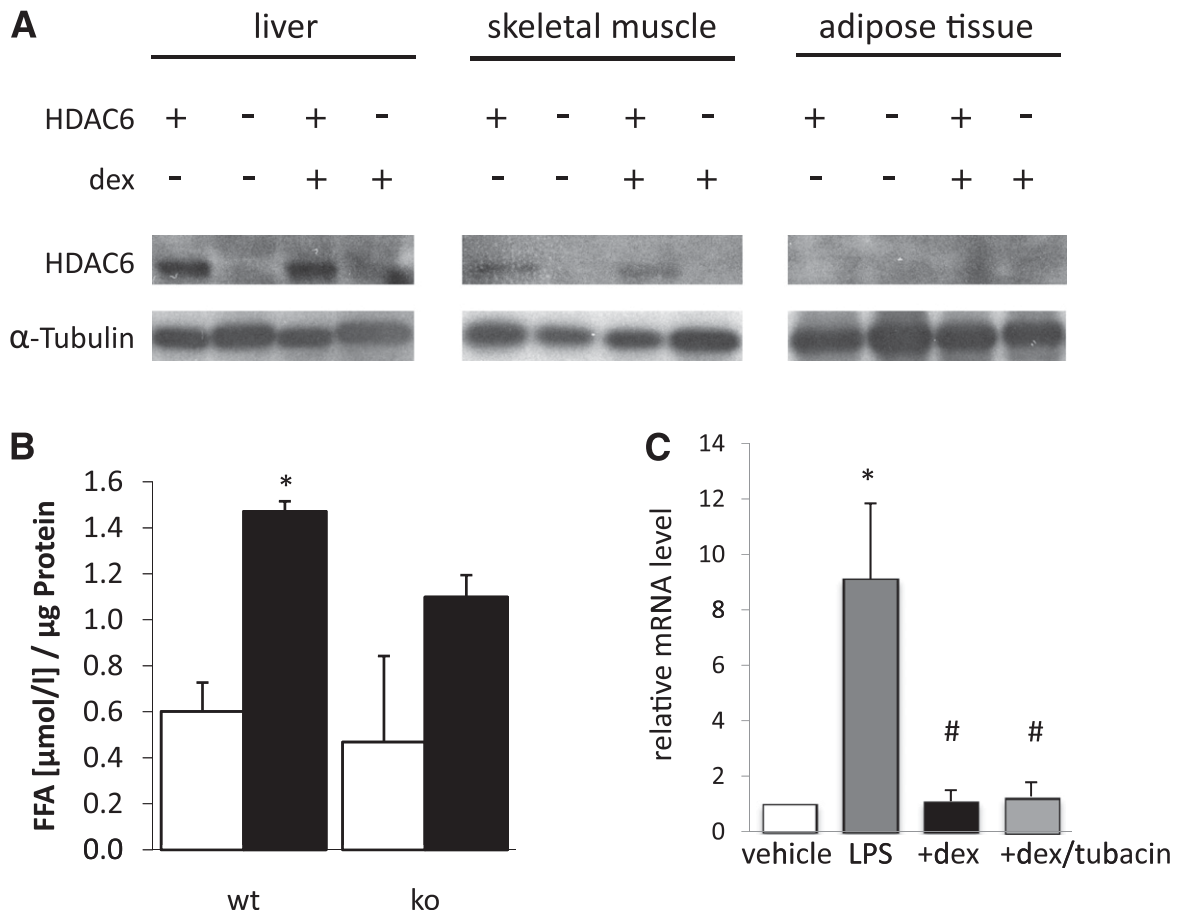

FIG. 5. Regulation of glucocorticoid-mediated actions by HDAC6 in adipose tissue and monocytes. *A*: Western immunoblotting of HDAC6 in liver, skeletal muscle, and gonadal adipose tissue from wt (+) and HDAC6KO (−) mice injected with vehicle (−) or dexamethasone (+) according to the protocol described in RESEARCH DESIGN AND METHODS. *B*: Ex vivo lipolysis (free fatty acid [FFA] release) from gonadal fat pads injected with vehicle (□) or dexamethasone (■) (100 nmol/L). *n* = 5 wt and HDAC6KO mice were studied. **P* < 0.05 vs. wt plus vehicle. *C*: *IL-6* mRNA expression in human THP-1 cells after stimulation with vehicle or 10 ng/mL LPS for 13 h followed by treatment with dexamethasone (50 nmol/L) with or without tubacin (20 μmol/L). **P* < 0.05 vs. vehicle, #*P* < 0.05 vs. LPS alone.

the composition of corresponding regulatory multiprotein complexes.

Although HDACs in general are known for modulating gene transcription by deacetylating Lys residues of histones, HDAC6 as a predominantly cytoplasmic deacetylase was not expected to have major effects on gene transcription. However, presence of HDAC6 is not exclusively cytoplasmic. Westendorf et al. (35) reported that *Runx2/Cbfa1* interacts with HDAC6 and recruits it to the nuclei of osteoblasts regulating p21 gene transcription. In addition, HDAC6 has been described as a cofactor of the nuclear receptor coregulator *LCOR* involving HDAC6 corecruitment to target promoters (34). Together, these studies suggest a nuclear function of HDAC6 as a transcriptional coregulator under certain circumstances. In the current study, we were able to show that HDAC6 deficiency results in enhanced histone acetylation of the *PEPCK* promoter in the liver, corroborating its function as a nuclear coregulator. Thus, the loss of HDAC6 in the liver results in an attenuation of glucocorticoid-induced gene expression via suppression of glucocorticoid receptor translocation, which may be counterregulated by a parallel hyperacetylation of histones associated with the target promoter and activation of gene transcription. Since we did not observe a significant increase of basal *PEPCK* expression in HDAC6KO mice, it seems that for basal *PEPCK* gene regulation other histones may be involved, balancing the hyperacetylation of H3 and H4. However, our data support the general idea that HDAC6 is able to act as a hepatic histone deacetylase, potentially counterbalancing its action on glucocorticoid receptor translocation. A systematic analysis of the nuclear and cytosolic importance of HDAC6 for hepatic glucocorticoid/glucocorticoid receptor–mediated gene transcription is currently ongoing. Nevertheless, a bimodal action of HDAC6 (histone/HSP90 deacetylase) may provide another explanation for the moderate effects of HDAC6 deficiency on glucocorticoid-induced gene expression in the liver and identifies HDAC6 as a molecule involved in the fine-tuning of glucocorticoid receptor actions.

Tubacin is a specific small-molecule inhibitor of HDAC6 activity (30). Here, we show that tubacin significantly diminishes dexamethasone-induced expression of gluconeogenic genes. Tubacin has been characterized as blocking only the tubulin deacetylase domain of HDAC6 (30). Since HDAC6-dependent regulation of metabolic glucocorticoid receptor actions in the liver likely involves HSP90 and does not involve tubulin, our data seem to be surprising. However, it has recently been shown that HDAC6 requires both functional domains for its full deacetylating activity (42). Along this line, tubacin has recently been shown to also induce hyperacetylation of HSP90 by interacting with HDAC6, in

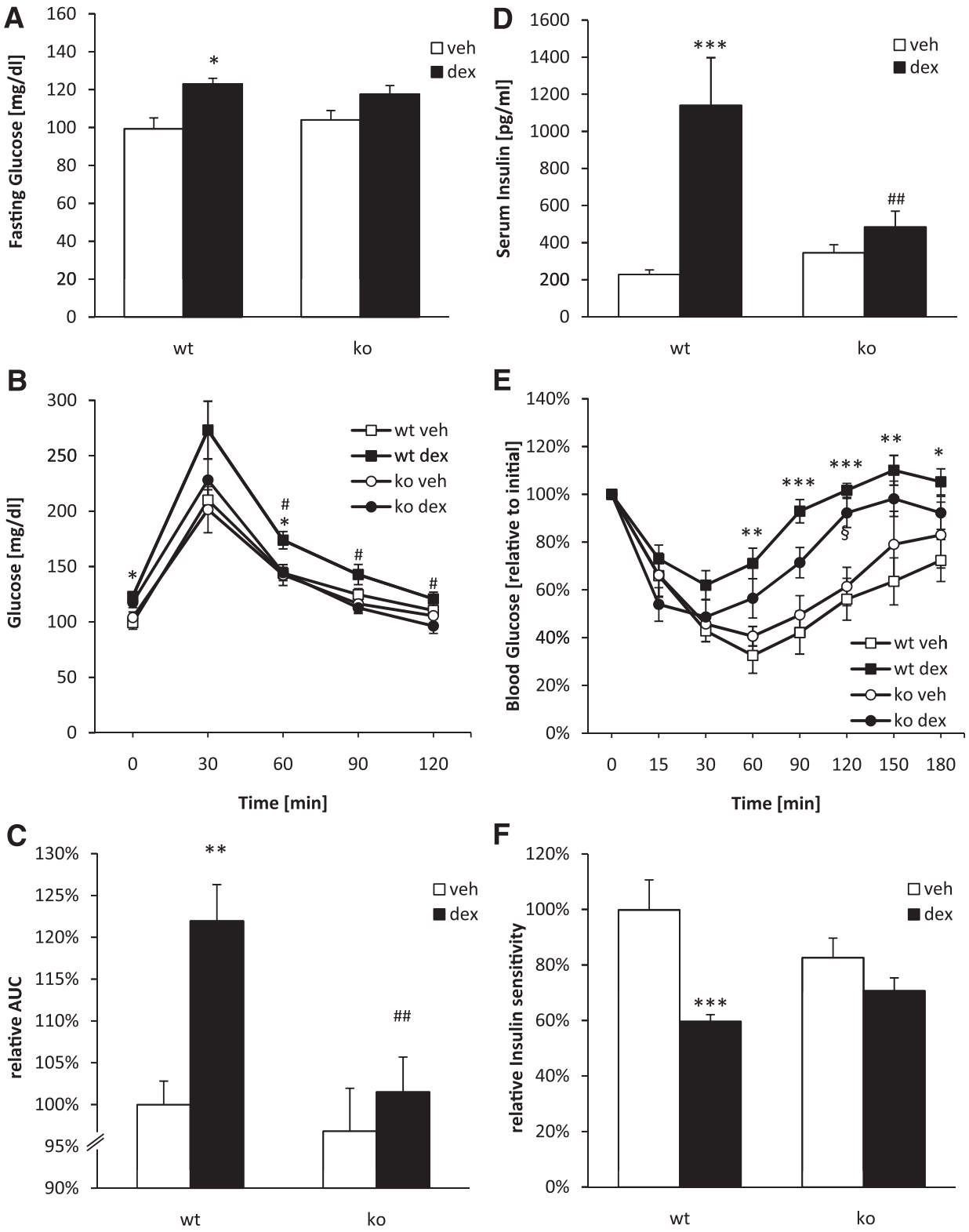

FIG. 6. Dexamethasone (dex)-induced impaired glucose tolerance and insulin resistance are attenuated in HDAC6KO (ko) mice. *A*: Blood glucose values determined in wt and HDAC6KO mice after an overnight fast. *B*: GTT. After 3 weeks of treatment, mice were given an injection of 1 mg glucose/kg body wt i.p. after overnight fasting. *C*: Corresponding AUC. *D*: Serum insulin in the fed state. *E*: ITT. After 4 weeks of treatment, fasted mice were given an injection of 0.5 units of insulin/kg body wt i.p. Results represent blood glucose concentration as a percentage of the initial glucose value. *F*: Corresponding AUC. Data represent means ± SEM (n = 5–9). *P < 0.05, **P < 0.01, ***P < 0.001 vs. wt plus vehicle (veh), #P < 0.05 vs. HDAC6KO plus dexamethasone, ##P < 0.01 vs. wt plus dexamethasone, §P < 0.05 vs. HDAC6KO plus vehicle.

addition to its actions on tubulin acetylation (18). These data support the potential of tubacin as a pharmacological intervention to inhibit HDAC6 activity resulting in HSP90 hyperacetylation, modifying hepatic glucocorticoid receptor function and potentially preventing prodiabetogenic glucocorticoid actions. A prerequisite for a therapeutic use of selective HDAC6 inhibitors during glucocorticoid treatment would be the preservation of glucocorticoid's anti-inflammatory actions. In the current study, we show that tubacin did not have a major impact on glucocorticoid-mediated suppression of LPS-induced inflammatory responses in THP-1 monocytes. These data may serve as a first hint that anti-inflammatory glucocorticoid actions can be preserved under HDAC6 inhibition. However, additional studies are indeed required to understand more comprehensively the role of HDAC6 on the glucocorticoid–glucocorticoid receptor axis in inflammatory cells.

Glucocorticoids influence hepatic gluconeogenesis by upregulating the key genes *PEPCK*, *PCX*, *FBP*, and *G6P*, which catalyzes the final step in this glucose-producing pathway (7). *PEPCK* had long been regarded as the rate-limiting step of gluconeogenesis until Burgess et al. (43) demonstrated that a 90% reduction of hepatic *PEPCK* only led to a 40% reduction in gluconeogenic flux. These data question the role of *PEPCK* as the central regulator of gluconeogenesis. Therefore, we analyzed the regulation of four genes involved in the gluconeogenic pathway. Glucocorticoid-induced *PEPCK* and *PCX* expression was only moderately affected by loss of HDAC6. In contrast, dexamethasone-mediated *FBP* and *G6P* induction was almost completely abrogated in HDAC6KO livers. More importantly, we were able to show that glucocorticoid-induced glucose output in isolated hepatocytes was attenuated in HDAC6KO hepatocytes, suggesting that even with a moderate modulation of *PEPCK*/*PCX* gene expression, modification of other gluconeogenic genes by HDAC6 is sufficient for regulating glucocorticoid-induced glucose output.

Enhanced gluconeogenesis contributes to the prodiabetogenic actions of glucocorticoids and results in hyperglycemia (7). Here, we show that HDAC6 deficiency protects against glucocorticoid-induced fasting hyperglycemia, as well as impaired glucose tolerance. That this observation is linked to the modification of the hepatic glucocorticoid–glucocorticoid receptor axis is supported by previous studies demonstrating that inactivation of the glucocorticoid receptor in liver improves systemic glucose metabolism in streptozotocin-induced diabetes (44). Furthermore, we observed that HDAC6 deficiency partially prevents dexamethasone-induced insulin resistance. However, the impact of HDAC6 on glucocorticoid actions in adipose tissue and monocytes was attenuated compared with the liver. This might result from distinct HDAC6 expression levels in different tissues or from its function as a histone deacetylase with preferences for distinct gene promoters. In addition, tissue-specific preference may provide an explanation for the incomplete restoration of dexamethasone-induced insulin resistance, since in selected tissues the adverse action of glucocorticoid on insulin/glucose/lipid metabolism is still present in HDAC6KO mice. Finally, since we did not directly investigate the role of HDAC6 for glucocorticoid/glucocorticoid receptor actions in muscle and pancreas, it might still be possible that these organs contributed to the observed phenotype. In relation to this, it has been shown that glucocorticoid treatment results in impairment of insulin-stimulated glucose uptake in skeletal muscle involving interactions with postreceptor insulin signaling (7,45). Future experiments are required to delineate the role of HDAC6 for muscular and pancreatic insulin/glucose metabolism.

In summary, the current study demonstrates that HDAC6 is an essential regulator of hepatic glucocorticoid-stimulated gluconeogenesis and impairment of whole-body glucose metabolism through modification of glucocorticoid receptor nuclear translocation. Since selective pharmacological inhibition of HDAC6 attenuates glucocorticoid-induced gluconeogenic gene expression, this intervention may provide a future therapeutic option against the prodiabetogenic actions of glucocorticoids.

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