Hexose-6-phosphate Dehydrogenase Knock-out Mice Lack 11β-Hydroxysteroid Dehydrogenase Type 1-mediated Glucocorticoid Generation*

The local generation of active glucocorticoid by NADPH-dependent, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) oxoreductase activity, has emerged as an important factor in regulating hepatic glucose output and visceral adiposity. We have proposed that this NADPH is generated within the endoplasmic reticulum by the enzyme hexose-6-phosphate dehydrogenase. To address this hypothesis, we generated mice with a targeted inactivation of the H6PD gene. These mice were unable to convert 11-dehydrocorticosterone (11-DHC) to corticosterone but demonstrated increased corticosterone to 11-DHC conversion consistent with lack of 11β-HSD1 oxoreductase and a concomitant increase in glucocorticoid clearance in vivo. This increased corticosterone clearance in the knock-out mice resulted in a reduction in circulating corticosterone levels. Our studies define the critical requirement of hexose-6-phosphate dehydrogenase for 11β-HSD1 oxoreductase activity and add a new dimension to the investigation of 11β-HSD1 as a therapeutic target in patients with the metabolic syndrome.

Corticosteroid hormone action is modulated in a tissue-specific fashion by the expression and activity of two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) that interconvert hormonally active cortisol (or corticosterone in rodents) to their inactive derivatives, cortisone/11-dehydrocorticosterone (11-DHC) (1). 11β-HSD2 acts as a NAD-dependent dehydrogenase, inactivating glucocorticoids and protecting the mineralocorticoid receptor from illegitimate activation by cortisol in mineralocorticoid receptor-rich tissues such as the kidney and colon (2).

By contrast, 11β-HSD1 is a bidirectional enzyme expressed in liver and adipose tissue. It catalyzes both oxidation and oxoreduction of glucocorticoids but acts in vivo predominantly as a NADPH-dependent oxoreductase (1). Independent of circulating glucocorticoid concentrations, the local generation of cortisol/corticosterone by 11β-HSD1 has emerged as an important factor in regulating hepatic glucose output (by augmenting gluconeogenesis) and visceral adiposity (by increasing adipocyte differentiation). Transgenic mice overexpressing 11β-HSD1 in liver and adipose tissue recapitulate features of the metabolic syndrome with visceral obesity, hepatic steatosis, glucose intolerance, insulin resistance, hyperlipidemia, and hypertension (3–5). In contrast, knock-out (KO) mice lacking 11β-HSD1 show improved glucose tolerance, enhanced insulin sensitivity, and reduced weight gain when fed a high fat diet (6, 7).

11β-HSD1 has therefore emerged as a novel therapeutic target to treat patients with obesity and insulin resistance. Indeed, selective 11β-HSD1 inhibitors improve glucose tolerance in diabetic mice (8–10).

These in vivo findings are dependent upon the oxoreductase activity of 11β-HSD1, which is puzzling because activity studies performed on the purified 11β-HSD1 enzyme or on cell/tissue homogenates indicate almost exclusive unidirectional dehydrogenase activity (11). We hypothesized that the enzyme hexose-6-phosphate dehydrogenase (Hex-6-PDH) is a key factor in conferring oxoreductase activity upon 11β-HSD1. Hex-6-PDH is a bifunctional enzyme that catalyzes the first two steps of an endolymacial pentose phosphate pathway (12) but is distinct from its cytosolic homologue, glucose-6-phosphate dehydrogenase, in being localized exclusively to the endoplasmic reticulum (ER) lumen. Hex-6-PDH has broad substrate specificity, accepting a variety of hexose 6-phosphates (13, 14) and has dual specificity for NAD+ and NADP+. However, under physiological conditions, the substrates within the ER are thought to be glucose 6-phosphate (Glc-6-P) and NADP+. Supply of Glc-6-P is ensured by the glucose 6-phosphate-transporter of the ER, but because the ER membrane is relatively impermeable to pyridine nucleotides, supply of NADP+ is maintained through a functional cooperation with endolymacial reductases such as 11β-HSD1.

Rare patients have apparent cortisone reductase deficiency, with decreased urinary excretion of cortisol metabolites relative to those of cortisone. Affected females have increased adrenal androgen levels and a clinical presentation resembling polycystic ovarian syndrome. Although it has been proposed that cortisone reductase deficiency has a digenic etiology, requiring mutations in both the HSD11B1 and H6PD genes (15), subsequent studies have demonstrated that polymorphisms in these genes are relatively common and are associated with neither abnormalities in urinary excretion nor an increased risk of polycystic ovarian syndrome (16, 17). Subsequently, a series of in vitro studies have demonstrated close cooperativity between 11β-HSD1 and Hex-6-PDH in preparations of rat liver microsomes with manipulation of Hex-6-PDH expression directly altering the set point of 11β-HSD1 activity (18–21). To date, however, little direct in vivo evidence exists as...
to the effects of Hex-6-PDH upon 11β-HSD1 activity. Here, through targeted gene inactivation in mice, we demonstrate that Hex-6-PDH inactivation profoundly affects 11β-HSD1 enzyme activity.

EXPERIMENTAL PROCEDURES

Reagents—Glucose 6-sulfate, Glc-6-P, NADP⁺, NADPH, Triton X-100, corticosterone, and 11-dehydrocorticosterone were purchased from Sigma-Aldrich. All restriction enzymes and materials used for conventional cloning methods were purchased from New England Biolabs (Ipswich, MA). Tritiated corticosterone (specific activity, 1 mCi/ml) was purchased from Amersham Biosciences and used to generate radiolabeled 11-DHC as described (22).

Design—All animal experiments had the approval of the Institutional Animal Care and Use Committee and were performed according to procedures approved by that committee. Mice were housed in standard conditions on a 12-h light/12-h dark cycle with access to standard rodent chow and water ad libitum.

Generation of a Targeted H6PD Gene—The murine H6PD gene spans ~15 kb and contains 5 exons. Genomic DNA from 129SvJ embryonic stem cells was used to amplify 5.5-kb 5' and 2-kb 3' homology arms, which were subsequently cloned into pBluescript SK(+) containing both PGK-Neo and thymidine kinase cassettes. The targeting vector was designed to replace exons 2 and 3. Following verification by DNA sequencing, the construct was linearized with SacII and electroporated into E14TG2a mouse embryonic stem cells. Southern hybridization of Ncol-digested genomic DNA probed with a 500-bp H6PD fragment located at the 5' and 3' ends of the targeting vector identified cells positive for a recombined H6PD locus after selection in G418 and gan-cyclovir. Three targeted embryonic stem cell clones were expanded, screened with Neo and 3' probes, and karyotyped to ensure correct recombination and chromosomal integrity. Two clones were injected into C57BL/6 blastocysts to produce chimeric mice. Chimeric mice derived from embryonic stem cell clone were mated with C57BL/6 females to achieve germ line transmission of the mutant allele. From these, heterozygote mice were intercrossed to generate WT, heterozygotes, and WT females to achieve germ line transmission of the mutant allele. From these, double heterozygote mice were intercrossed to generate KO mice.

Hexose-6-phosphate Dehydrogenase Knock-out Mouse

Disruption of the Mouse H6PD Gene—A null mutation was created in the murine H6PD gene through replacement of exons 2 and 3 with a neomycin resistance cassette via homologous recombination (Fig. 1A). Recombination was detected in 8 of 110 clones screened by Southern blotting, and correct targeting fidelity was confirmed with further Southern blotting using the 5' and neomycin probes. Homozygous KO mice were bred and maintained through intercrosses of 129SvJ and 129SvJ.
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C57BL/6 heterozygote mice and detected by Southern blotting using the 3' probe (Fig. 1B). Mice were genotyped by PCR using DNA extracted from tail biopsy. Primers P1, P2, and P3 were used in a multiplex PCR reaction to rapidly characterize WT, KO, and heterozygote mice. Gestation time and litter size were normal in heterozygote crosses, with genotyping of 178 animals revealing no deviation from a Mendelian distribution of alleles. No gross morphological abnormalities were seen in KO mice at birth.

Knock-out Mice Lack Hex-6-PDH Protein and Activity—Immunoblot analysis showed that no Hex-6-PDH protein could be detected in KO liver microsomes (Fig. 1C). Hepatic Hex-6-PDH enzyme activity in WT and KO mice was assessed using glucose 6-sulfate as a specific substrate for murine Hex-6-PDH (26). No Hex-6-PDH activity was measurable in KO mice (Fig. 2) compared with 5.5 ± 0.41 nM NADPH/mg/min in WT mice and 3.2 ± 0.35 nM NADPH/mg/min in heterozygous mice.

Set Point of 11β-HSD1 Is Altered in Hex-6-PDH KO Mice—11β-HSD1 enzyme function was assessed by examining the oxoreductase and dehydrogenase activities in liver microsomes from WT and KO mice either in the presence or absence of Glc-6-P, the substrate for Hex-6-PDH (Fig. 3). In the absence of Glc-6-P, intact microsomes from WT mice showed 7.1 ± 1.8% conversion of 11-DHC to corticosterone (oxoreductase activity) and 10.3 ± 1.3% conversion of corticosterone to 11-DHC (dehydrogenase activity). In the presence of Glc-6-P, conversion of 11-DHC to corticosterone increased to 31.8 ± 6.8% in WT mice. By contrast, liver microsomes from KO mice consistently gave ≤5% conversion of 11-DHC to corticosterone, with or without Glc-6-P, indicative of absence 11β-HSD1 oxoreductase activity (Fig. 3A). This loss in oxoreductase activity in KO animals was mirrored by an increase in dehydrogenase activity (conversion of corticosterone to 11-DHC 29.4 ± 0.5% in KO versus 10.3 ± 1.3% in WT), thus indicating a switch from oxoreductase to dehydrogenase activity in the absence of Hex-6-PDH.

Permeabilization of WT microsomes with detergent resulted in a loss of conversion of 11-DHC to corticosterone regardless of whether substrate for Hex-6-PDH (Glc-6-P) was absent or present (1.6 ± 0.7% without Glc-6-P, 3.9 ± 1.3 with Glc-6-P), indicating the requirement for

![Figure 1](targeted_disruption_of_murine_H6PD.png)

**FIGURE 1.** Targeted disruption of murine H6PD. A, schematic representation of the 5 exons of the H6PD gene, the targeting vector, and recombined allele. The location of the 5' and 3' external probes used for Southern hybridization and PCR primers P1, P2, and P3 used for genotyping are indicated. The recombined allele has exons 2 and 3 replaced with a phosphoglycerokinase-driven neomycin resistance cassette. N, NsiI; Nc, NcoI; NEO, neomycin resistance cassette; TK, thymidine kinase negative selection cassette. B, successful targeting of H6PD in embryonic stem cells and tail DNA from germ-line mice was confirmed by Southern hybridization of NcoI-digested genomic DNA extracted from tail sections. HET, heterozygote; C, immunoblot analysis of mouse microsomal liver protein. A band of ~89 kDa was visible from WT liver microsomal protein but absent in KO.

![Figure 2](hex-6-pdh_enzyme_assay.png)

**FIGURE 2.** Hex-6-PDH enzyme assay. Hex-6-PDH activity was determined spectrophotometrically by the production of NADPH at 340 nm when the reaction contained the Hex-6-PDH-specific substrate glucose 6-sulfate, with readings taken at 1-min intervals for 5 min. KO microsomes were devoid of activity, whereas activity was attenuated by ~50% in heterozygotes (HET) (0 nM NADPH/mg/min for KO mice versus 5.5 ± 0.41 nM NADPH/mg/min for WT and 3.2 ± 0.35 nM NADPH/mg/min for heterozygotes; n = 4).
intact microsomes and presumably a close, cooperative environment between Hex-6-PDH and 11β-HSD1 in the ER to ensure 11-oxoreductase activity.

An assessment of 11β-HSD1 activity in vivo was inferred through GC/MS analysis of steroid metabolites from pooled urine collections from male and female mice (n = 11005 in each group). Tetrahydro- and hexahydrocorticosterone and 11-DHC metabolites were found in mouse urine. These steroids were the only compounds with known mass spectra allowing accurate structures to be assigned. The dominant stereochemistry of the tetrahydro-11-DHC and tetrahydrocorticosterone metabolites was 3α,5β, and the hexahyrometabolites were predominately 3α,5β,20α.

WT mice almost exclusively excrete steroids with a 11β-hydroxyl group, and thus only minor amounts of 11-DHC metabolites were detected. Conversely, the dominant steroids in the KO mice were 11-DHC metabolites. In male WT mice the ratio of 11-DHC metabolites to corticosterone metabolites was 0.06, increasing to 9.3 in the KO animals. In female WT mice the 11-DHC/corticosterone ratio was 0.04, increasing to 1.83 in KO animals. When expressed as a percentage of 11-DHC metabolites in the urine of WT and KO animals, the marked differences confirmed the lack of oxoreductase activity in KO mice (Fig. 4).

**DISCUSSION**

Our data show that the inactivation of the murine H6PD gene dramatically alters the reaction direction of 11β-HSD1. In isolated but intact WT microsomal preparations, there was minimal oxoreductase activity and moderate dehydrogenase activity. However, upon addition
of Glc-6-P (the intracellular substrate for Hex-6-PDH), oxoreductase activity was stimulated ~6-fold, whereas dehydrogenase activity was unaffected. Upon permeabilization of the microsomes, this stimulation of reductase activity by Glc-6-P was lost, presumably because of the disruption of an intimate interaction between 11β-HSD1 and Hex-6-PDH proteins. 11β-HSD1 is bound to the inner ER membrane (27), whereas Hex-6-PDH is free-floating within the ER lumen (28). Further studies are warranted to define the interaction between these two proteins within the ER lumen. By contrast, intact hepatic microsomes from Hex-6-PDH KO mice show minimal basal oxoreductase that cannot be stimulated with the addition of Glc-6-P (the intracellular substrate for Hex-6-PDH), oxoreductase (1, 2). Here, using a similar principle, the ratio of 11-dehydrosteroids (cortisol/cortisone) is an accurate reflection of 11β-HSD1 activity. It remains to be seen whether this alteration in 11β-HSD1 activity accounts for differences in the function of the hypothalamus-pituitary-adrenal axes between these mice.

Our study has focused upon what appears to be a crucial interaction between Hex-6-PDH and 11β-HSD1, largely in hepatocytes. However, Hex-6-PDH is ubiquitously expressed in mammalian tissues, including the liver, adrenal glands, spleen, kidney, heart, lungs, muscle, testes, ovaries, prostate, uterus, intestine, and placenta (30, 31). Despite an extensive search we have been unable to identify other endoluminal NADPH-dependent enzymes, but it is possible that these exist and will also be compromised. A detailed phenotypic analysis of these mice is now indicated, not only to define the effect of lack of glucocorticoid generation in liver and adipose tissue upon glucose tolerance and adiposity but also to investigate the consequences of disruption of ER redox potential in other key tissues.

In summary, these data further our understanding of the regulation and function of 11β-HSD1, demonstrating that NADPH-dependent oxoreductase activity is critically dependent on the activity of the enzyme Hex-6-PDH. Mice lacking Hex-6-PDH have a profound switch in 11β-HSD1 activity from oxoreductase to dehydrogenase, i.e. activation to inactivation. There is great interest in the role of 11-oxoreductase activity in the treatment of the metabolic syndrome, with selective 11β-HSD1 inhibitors demonstrating improved glucose tolerance and weight reduction in mouse models of obesity (8, 9). Our findings add a new complexity to this exciting pathway but also define a completely novel biochemical pathway that regulates the ER NADPH:NADP ratio and redox potential.

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REFERENCES

1. Tomlinson, J. W., Walker, E. A., Bujalska, I. J., Draper, N., Lavery, G. G., Cooper, M. S., Hewison, M., and Stewart, P. M. (2004) Endocrinology 25, 831–866
2. White, P. C., Mune, T., and Agarwal, A. K. (1997) Endocrinology 216, 135–156
3. Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R., and Flier, J. S. (2001) Science 294, 2166–2170
4. Masuzaki, H., Yamamoto, H., Kenyon, C. J., Elmqist, J. K., Morton, N. M., Paterson, J. M., Shinyama, H., Sharp, M. G., Fleming, S., Mullins, J. J., Seckl, J. R., and Flier, J. S. (2003) J. Clin. Invest. 112, 83–90
5. Paterson, J. M., Morton, N. M., Fievet, C., Kenyon, C. J., Holmes, M. C., Staels, B., Seckl, J. R., and Mullins, J. J. (2004) Proc. Natl. Acad. Sci. U S A 101, 7096–7093
6. Kotelclevsky, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R., Seckl, J. R., and Mullins, J. J. (1997) Proc. Natl. Acad. Sci. U S A 94, 14924–14929
7. Morton, N. M., Holmes, M. C., Fievet, C., Staels, B., Taffieux, A., Mullins, J. J., and Seckl, J. R. (2001) J. Biol. Chem. 276, 41293–41300
8. Alberts, P., Engblom, L., Edling, N., Forsgren, M., Klingstrom, G., Larsson, C., Ronquist-Nii, Y., Ohman, B., and Abrahamsson, L. (2002) Diabetologia 45, 1528–1532
9. Alberts, P., Nilsson, C., Selen, G., Engblom, L. O., Edling, N. H., Norling, S., Klingstrom, G., Larsson, C., Forsgren, M., Ashkhari, M., Nilsson, C. E., Fieder, M., Bergquist, E., Ohman, B., Biorckstrand, E., and Abrahamsson, L. B. (2003) Endocrinology 144, 4755–4762
10. Barf, J., Vallgards, J., Emond, R., Hagstrom, C., Kurz, G., Nygren, A., Larwood, V., Mostialou, A., Axelson, K., Nilsson, R., Engblom, L., Edling, N., Ronquist-Nii, Y., Ohman, B., Alberts, P., and Abrahamsson, L. (2002) J. Med. Chem. 45, 3813–3815
11. Walker, E. A., Clark, A. M., Hewison, M., Ride, J. P., and Stewart, P. M. (2001) J. Biol. Chem. 276, 21343–21350
12. Mason, P. J., Stevens, D., Diez, A., Knight, S. W., Scopes, D. A., and Vulliamy, T. J. (1999) Blood Cells Mol. Dis. 25, 30–37
13. Beutler, E., and Morrison, M. (1967) J. Biol. Chem. 242, 5289–5293
14. Romanelli, A., Stamnen, F., Vidal, H., Tchu, S., and van de W. G. (1994) Biochem. Biophys. Res. Commun. 200, 1491–1497
15. Draper, N., Walker, E. A., Bujalska, I. J., Tomlinson, J. W., Chalder, S. M., Arlt, W., Lavery, G. G., Bedendo, O., Ray, D. W., Laing, I., Malinowicz, E., White, P. C., Hewison, M., Mason, P. J., Connell, J. M., Shackleton, C. H., and Stewart, P. M. (2003) Nat. Genet. 34, 434–439
16. San Millan, J. L., Botella-Carretero, J. I., Alvarez-Blasco, F., Luque-Ramirez, M., Sanchez, J., Moggetti, P., and Escobar-Morreale, H. F. (2005) J. Clin. Endocrinol. Metab.
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90, 4157–4162
17. White, P. C. (2005) J. Clin. Endocrinol. Metab. 90, 5880–5883
18. Atanasov, A. G., Nashev, L. G., Schweizer, R. A., Frick, C., and Odermatt, A. (2004) FEBS Lett. 571, 129–133
19. Banhegyi, G., Benedetti, A., Fulceri, R., and Senesi, S. (2004) J. Biol. Chem. 279, 27017–27021
20. Bujalska, I. J., Draper, N., Michailidou, Z., Tomlinson, J. W., White, P. C., Chapman, K. E., Walker, E. A., and Stewart, P. M. (2005) J. Mol. Endocrinol. 34, 675–684
21. McCormick, K. L., Wang, X., and Mick, G. J. (2006) J. Biol. Chem. 281, 341–347
22. Agarwal, A. K., Tusie-Luna, M. T., Monder, C., and White, P. C. (1990) Mol. Endocrinol. 4, 1827–1832
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Shackleton, C. H. (1993) J. Steroid Biochem. Mol. Biol. 45, 127–140
25. Shackleton, C. H. L., Merdink, J, and Lawson, A. M. (1990) Steroids and Bile Acid Analyses. Mass Spectrometry in Biological Materials (McEwen, C., ed) pp. 297–377, Marcel Dekker, New York
26. Clarke, J. L., and Mason, P. J. (2003) Arch. Biochem. Biophys. 415, 229–234
27. Odermatt, A., Arnold, P., Stauffer, A., Frey, B. M., and Frey, F. J. (1999) J. Biol. Chem. 274, 28762–28770
28. Ozols, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5302–5306
29. Gerin, I., and Van Schaftingen, E. (2002) FEBS Lett. 517, 257–260
30. Tanahashi, K., and Hori, S. H. (1980) J. Histochem. Cytochem. 28, 1175–1182
31. Mandula, B., Srivastava, S. K., and Beutler, E. (1970) Arch. Biochem. Biophys. 144, 155–161