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Hexose-6-Phosphate Dehydrogenase and 11β-Hydroxysteroid Dehydrogenase-1 Tissue Distribution in the Rat

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Intracellular concentrations of the glucocorticoids cortisol and corticosterone are modulated by the enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2. 11β-HSD1 is a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent microsomal reductase that converts the inactive glucocorticoids cortisone and 11-dehydrocorticosterone to their active forms, cortisol and corticosterone. Hexose-6-phosphate dehydrogenase (H6PDH) is an enzyme that generates NADPH from oxidized NADP (NADP$^+$) within the endoplasmic reticulum. In the absence of NADPH or H6PDH to regenerate NADPH, 11β-HSD1 acts as a dehydrogenase and inactivates glucocorticoids, as does 11β-HSD2. A monoclonal antibody against H6PDH was produced to study the possibility that 11β-HSD1 in the absence of H6PDH may be responsible for hydroxysteroid dehydrogenase activity in tissues that do not express significant amounts of 11β-HSD2. H6PDH and 11β-HSD1 expression was surveyed in a variety of rat tissues by real-time RT-PCR, Western blot analysis, and immunohistochemistry. H6PDH was found in a wide variety of tissues, with the greatest concentrations in the liver, kidney, and Leydig cells. Although the brain as a whole did not express significant amounts of H6PDH, some neurons were clearly immunoreactive by immunohistochemistry. H6PDH was amply expressed in most tissues examined in which 11β-HSD1 was also expressed, with the notable exception of the renal interstitial cells, in which dehydrogenase activity by 11β-HSD1 probably moderates activation of the glucocorticoid receptor because rat renal interstitial cells do not have significant amounts of mineralocorticoid receptors. This antibody against the H6PDH should prove useful for further studies of enzyme activity requiring NADPH generation within the endoplasmic reticulum. (*Endocrinology* 149: 525–533, 2008)

BINDING OF CORTISOL and corticosterone to the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) is modulated by the presence of the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and 2 enzymes.

11β-HSD1 can act as a dehydrogenase (oxidase) or a reductase, however, it is primarily a reductase *in vivo* and in intact cells, and converts the inactive metabolites cortisone and 11-dehydrocorticosterone to the glucocorticoids cortisol and corticosterone. 11β-HSD1 is expressed in many rat tissues, most prominently in the liver, lung, proximal tubules of the renal cortex and interstitial cells of the renal medulla and papilla (in the rat, but not human kidney), gastric parietal cells, and testis (1–5). 11β-HSD1 does not colocalize with the MR in the kidney (6).

11β-HSD1 is thought to be anchored in the membrane of the endoplasmic reticulum (ER) with its catalytic site within the ER lumen (7–9). Its reductase activity requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) (10). Most NADPH is produced by the oxidation of phosphorylated hexoses by the cytosolic enzyme, glucose-6-phosphate dehydrogenase that catalyzes the first step in the pentose phosphate pathway. However, NADPH does not freely cross the microsomal membrane. Hexose-6-phosphate dehydrogenase (H6PDH) is a microsomal enzyme that catalyzes the first two steps of the pentose phosphate pathway to generate NADPH from oxidized NADP (NADP$^+$) within the ER (10, 11). H6PDH has been found in a wide variety of tissues, particularly those involved in detoxification and steroid metabolism, most prominently the liver, testes, and placenta (10, 12). The direction of 11β-HSD1 activity is dependent upon the coexpression H6PDH to generate the cofactor NADPH; without H6PDH, 11β-HSD1 acts as a dehydrogenase and inactivates glucocorticoids, as does 11β-HSD2 (5, 10, 13). H6PDH knockout mice have no 11β-HSD1 reductase activity (14)

11β-HSD2 is an oxidized nicotinamide adenine dinucleotide dependent dehydrogenase with Michaelis-Menten constants (kMs) for cortisol and corticosterone low enough to be relevant to circulating levels of free glucocorticoids (15, 16). It has been cloned for several species and demonstrated in both epithelial and nonepithelial tissues (17–19). Important exceptions are the adult heart and most areas of the brain (20). Its expression in the central nervous system is very

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Abbreviations: aa, Amino acids; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; H6PDH, hexose-6-phosphate dehydrogenase; 11β-HSD, 11β-hydroxysteroid dehydrogenase; ihc, immunohistochemistry; MR, mineralocorticoid receptor; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SDS, sodium dodecyl sulfate; STF, Streck's Tissue Fixative.

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limited (21–23), but the ready conversion of corticosterone to 11-dehydrocorticosterone has been documented in the brain (24). Despite the fact that 11β-HSD2 has not been demonstrated in the adult heart, aldosterone activates MR in the heart, both in hyperaldosteronism and congestive heart failure (25–28).

It is not certain how the MR can be occupied and activated by aldosterone in cells in which the 11β-HSD2 is not coexpressed with the receptor. Several mechanisms for which there is circumstantial but inconclusive evidence have been proposed, including the existence of an as yet uncharacterized steroid dehydrogenase (29–32), paracrine or autocrine action of locally synthesized aldosterone (33, 34), and local synthesis of a more potent metabolite of aldosterone (35, 36). However, another potential mechanism for 11β-HSD activity in the absence of the 11β-HSD2 is hydroxysteroid dehydrogenase activity by the 11β-HSD1 in the absence of sufficient H6PDH activity (10). In this study we have measured the expression of the mRNA and protein of 11β-HSD1 and H6PDH in a variety of tissues by real-time PCR, Western blot analysis, and immunohistochemistry (ihc).

Materials and Methods

Tissues were harvested from normal 3-month-old female and male Sprague Dawley rats consuming a standard rat diet (maintenance rodent chow; Harlan Teklad, Indianapolis, IN) and tap water *ad libitum*. Husbandry and all procedures followed the National Research Council Guide for the Care and Use of Laboratory Animals and were performed in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. The animal care and use protocol for the current studies was approved by the Jackson Veterans Affairs Medical Center Institutional Animal Care and Use Committee. The rats were anesthetized with isoflurane in oxygen, and a variety of tissues (Figs. 1 and 2) were either quickly removed and frozen in liquid nitrogen for RNA and protein isolation, or the animals were perfused first with heparinized saline, then Streck's Tissue Fixative (STF) (Streck Laboratories, La Vista, NE) before tissue harvest.

Real-time RT-PCR

Total RNA was extracted with Tri-Reagent (Molecular Research Center, Cincinnati, OH), resuspended in diethyl pyrocarbonate-H_2O, DNase treated with Turbo DNAfree kit (Ambion, Austin, TX), and quantified by spectrophotometry. Five micrograms of RNA were reverse transcribed with 0.5 μg T_{12}VN primer and Superscript III (Invitrogen, Carlsbad, CA) in a final volume of 20 μl. The reaction was performed for 60 min at 50 C and terminated by incubation at 75 C for 15 min. Primers for H6PDH (sense: 5′-TTTCTGCAGCTGAGCCAGTA-3′; antisense: 5′-CTGGGTCTCGATGTCCTTGT-3′, product size: 78 bp), 11β-HSD1 (sense: 5′-GCAGACCGATTTGTTGTTGA-3′; antisense: 5′-GTGGATATCATCGTGGAAGAGAG-3′, product size: 108 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: 5′-AAGATGGTGAAGGTCGGTGT-3′; antisense: 5′-GTTGATGGCAACAATGTCCACT-3′, product size: 99 bp) were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and checked for the absence of cross-reactivity by BLAST

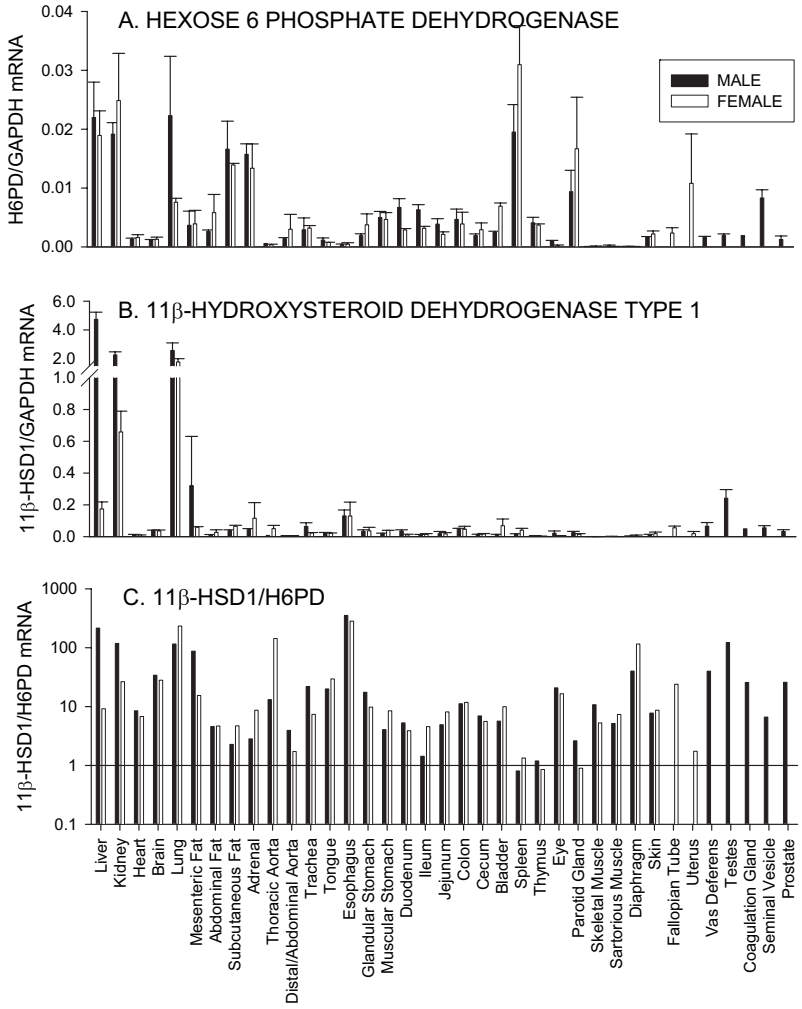

FIG. 1. A, The ratio of mRNA for H6PDH and GAPDH for a variety of tissues for male and female rats. B, The ratio of mRNA for 11β-HSD1 and GAPDH for a variety of tissues for male and female rats. C, The ratio of mRNA for 11β-HSD1 and H6PDH on a log scale.

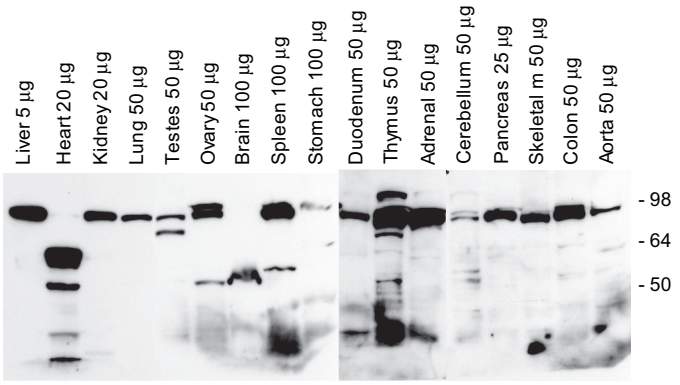

FIG. 2. Representative Western blots using varying amounts of total cellular protein of different tissues detected by the antibody against H6PDH at approximately 96 kDa.

search. Real-time PCR contained 1 µl reverse transcribed product, 0.1 µM each of primer, 0.2 mM deoxyribonucleotide triphosphates, SYBR green I (1:20,000 final concentration; Molecular Probes, Eugene, OR), and 1 µl titanium *Taq* DNA polymerase (Clontech, Palo Alto, CA). Cycling conditions were 1 min at 95 C, followed by 50 cycles of 15 sec at 95 C, 15 sec at 60 C, and 1 min at 72 C. The PCR was performed in an i-Cycler thermal cycler (Bio-Rad Laboratories, Hercules, CA), and real-time data were collected during the extension phase of the PCR cycle. After PCR amplification, the specificity of the PCR was confirmed by melting temperature determination of the PCR product and electrophoretic analysis in 2% agarose gels. For the standard curve, PCR products were cloned in the pCR2.1 TOPO vector (Invitrogen Life Technologies, Inc.) and were diluted to add $10–10^8$ molecules per PCR. Results are expressed as molecules of gene of interest standardized by molecules of GAPDH.

Antibodies. Polyclonal antibodies were raised in sheep against a portion of the human H6PDH comprising amino acids (aa) 757–770 (C-LVSRVGHEPKKWPI) and 215–229 (C-LPFRDQNRKALDGL), regions that are identical to the rat sequence. The peptides were conjugated to keyhole limpet hemocyanin using iodoacetamido-caproyl-N-hydroxysuccinimide as described previously (37). The antibodies were purified by affinity purification using the peptide conjugated to a Sulfolink column (Pierce, Rockford, IL). Results were similar for both H6PDH antibodies; the antibody against 757–770 aa was used for the figures except where noted (Fig. 3). The antibody against 11β-HSD1 was a rabbit polyclonal antibody designated RAH113 (2).

Western blot for 11β-HSD1 and H6PDH. Rat kidney microsomes were isolated by differential centrifugation as described before (38). Microsomes were solubilized using Laemmli buffer (39) and run in a 12% PAGE (11β-HSD) or 7.5% PAGE (H6PD), transferred by semidry blot to a polyvinylidene difluoride membrane, dried, blocked with 5% nonfat milk, and incubated with the anti-11β-HSD1 or sheep anti-H6PDH antibodies. The blots were then incubated with peroxidase-labeled donkey antirabbit or antisheep second antibodies and developed using West Pico Chemiluminescence substrate from Pierce.

ihc. After removal from the perfused animal, tissues were further fixed for 18–24 h in STF, then paraffin blocked for sectioning into 6-mm slices. Sodium dodecyl sulfate (SDS) 0.2% was used both in the blocking solution and primary antibody buffer to ensure antibody permeability and to unmask antigens. Immunocytochemistry was performed using sheep anti-H6PDH and rabbit anti-11β-HSD1 antibodies, followed by donkey antirabbit or antisheep biotin-labeled antibodies, and detected using streptavidin-peroxidase system (Zymed Laboratories, San Francisco, CA) and 3,3′-diaminobenzidine HCl (Sigma-Aldrich, St. Louis, MO.), then counterstained with Gil hematoxylin. Controls were done for every tissue using the two different secondary antibodies, without the primary antibodies.

Statistical analysis

Differences in the measured variables between control-treated samples were evaluated by ANOVA and expressed as the mean ± SEM where appropriate.

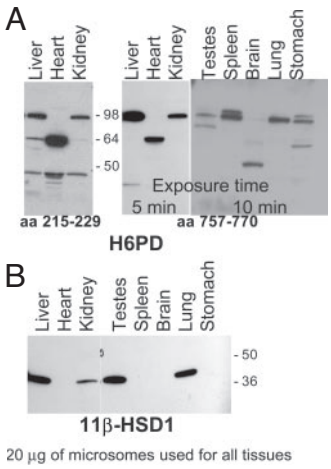

FIG. 3. A composite of Western blot gels using 20-µg microsomes for several tissues. A, The same gel was probed first with H6PDH aa 757–770 (*portion right of the molecular mass marks*), stripped, and reprobed with the H6PDH aa 215–229. Exposure times as labeled. B, 11β-HSD1.

Results

Figure 1A is a plot of the amount of H6PDH mRNA relative to that of GAPDH from female and male Sprague Dawley rats. H6PDH mRNA was detected in all tissues. Its expression was greatest in liver, kidney, lung, spleen, parotid gland, adipose tissue (both white and brown), uterus, and seminal vesicle. There were no significant gender differences in H6PDH mRNA expression in common tissues. Figure 1B is a plot of amounts of 11β-HSD1 mRNA relative to that of GAPDH. 11β-HSD1 mRNA was also detected in all tissues assayed. Males expressed more 11β-HSD1 relative to GAPDH mRNA than females in the liver, kidney, and mesenteric fat; females expressed more in the adrenal and bladder. Expression of 11β-HSD1 in the liver of male rats was over 20 times that of females, as has been reported previously (2). Although GAPDH is considered to be among the best of the commonly used housekeeping genes for the rat (40), Barber *et al.* (41) reported up to a 15-fold difference in the expression of mRNA for GAPDH between some human tissue GAPDH. Greater GAPDH expression in skeletal muscle would explain the relatively low values of H6PDH in this tissue (Fig. 1A), compared with protein expression by Western blot (Fig. 2). Therefore, the ratio of 11-HSD1 to H6PDH has been used to compare the gene expression of the two enzymes in each tissue (Fig. 1C).

Results of multiple Western blots for H6PDH protein in tissue homogenates and microsomes were similar with both H6PDH antibodies and concordant with those of the mRNA measurements for H6PDH. Figure 2 is a representative Western blot using different amounts of protein for different tissues and demonstrates relative levels of protein expression detected by the antibody against aa 757–770. The H6PDH antibody detected a band in the expected molecular mass range of approximately 98 kDa in most tissues analyzed, with the largest amount consistently detected in the liver. The band at approximately 98 kDa for 5 µg liver protein was of similar or greater intensity than the bands for 20 µg kidney, 25 µg pancreas, 50 µg lung, testes, ovary, duodenum, thy-

mus, adrenal, skeletal muscle, colon, and aorta, and 100 μg spleen and stomach. The protein sample for the aorta comprised adhering tissue as well as vascular smooth muscle, an important consideration in interpreting these data, as discussed in the description of the ihc of the aorta. As expected from the relatively limited quantities of mRNA detected, bands at approximately 98 kDa for heart and brain were very faint or absent in multiple Western blots (Figs. 2 and 3, A and B; data not shown). Although no band at this molecular mass was detected in 100 μg whole brain in the gel (Fig. 3A), 50 μg cerebellum did produce a distinct, if faint, band at this molecular mass. Only a very faint band at approximately 98 kDa was detected in multiple heart samples, though the antibody consistently detected a very strong band at approximately 64 kDa and a fainter one at approximately 55 kDa in microsomal protein samples with both H6PDH antibodies (Fig. 3) and in other gels not shown. Similarly, prominent bands of lower molecular mass representing an unknown protein were detected in brain samples.

Figure 3 is a composite of Western blot gels using 20 μg microsomes for several tissues. The gel in Fig. 3A was probed first with the H6PDH antibody against aa 757–770 (portion to the right of the molecular mass marks), then stripped and reprobed with the H6PDH antibody against aa 215–229. Exposure time for the aa 215–229 H6PDH antibody was 45 sec; that for the same gel using the aa 757–770 H6PDH antibody, comprising the liver, heart, and kidney samples, was 5 min, and that for aa 757–770 for testes, spleen, brain, lung, and stomach was 10 min, allowing the detection of the smaller quantities of H6PDH protein in the testes, stomach, and lung. The gel in Fig. 3B represents protein detected by the anti-11β-HSD1 antibody. A very faint approximate 98-kDa band was detected for brain microsomal protein, but not for the heart. Bands of smaller molecular mass of about 64 and 50 kDa were detected consistently in heart and brain sample. Although these may be degradation products of the H6PDH, we do not know the nature of these proteins.

11β-HSD1 protein was clearly detected at the expected molecular mass of about 36 kDa by Western blot in 20 μg microsomes from liver, kidney, testes, and lung (Fig. 3B). As expected from the literature, analysis of a larger amount of whole brain, cerebellum and brainstem protein, and extending the development time for the gel demonstrated bands at the appropriate molecular mass for 11β-HSD1, at roughly half the intensity of the band for 5 μg liver protein (Western blot gels not shown). Faint bands of 36 kDa were seen in samples of heart, spleen, thymus, stomach, skeletal muscle, aorta, and adrenal gland when gels were exposed for longer times (data not shown). The 11β-HSD1 antibody detected prominent bands of 36 kDa for protein extracted from sc, omental, and brown fat (gels not shown).

Figures 4 and 5 are composites of photomicrographs of ihc for H6PDH and 11β-HSD1 in multiple tissues. The control immunostaining procedures omitting the primary antibodies, but using antisheep antibodies for the H6PDH and antirabbit for the 11β-HSD1, were unequivocally negative for every tissue, though not all are shown due to space considerations. Staining for both H6PDH and 11β-HSD1 was granular or punctuate as expected from their location within the ER. Both enzymes were highly and fairly uniformly expressed in the liver (Fig. 4, a–d) when 0.2% SDS was added to the blocking buffer to unmask antigenic sites. Cells comprising the walls of the vessels and bile ducts were not significantly stained by either antibody. Occasional macrophages were stained by both. If SDS was not added to the blocking and primary antigen buffers, staining for both enzymes was more intense in cells closest to the central veins and portal triads in a pattern reported previously (2, 42).

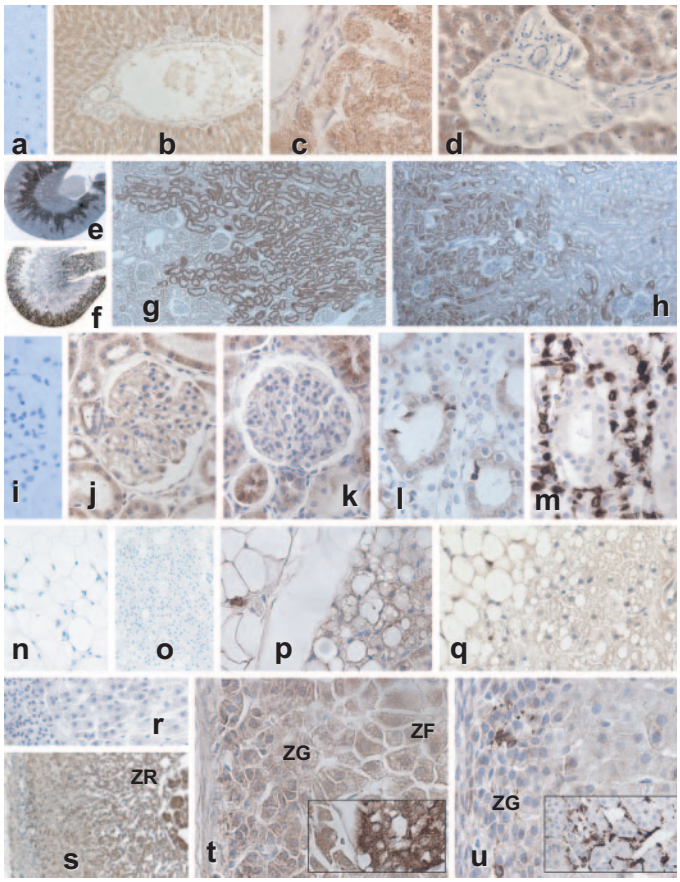

FIG. 4. Photomicrographs of rat tissues using antibodies against the *H6PDH* and 11β-HSD1 for ihc. a–d, Liver permeabilized with SDS. a, Control. b and c, H6PDH. d, 11β-HSD1. e–m, Kidney. e, H6PDH. f, 11β-HSD1. g, Outer medulla H6PDH. h, Outer medulla 11β-HSD1. i, Control. j, Glomerular H6PDH. k, Glomerulosa 11β-HSD1. l, Inner medulla H6PDH. m, Inner medulla 11β-HSD1. n–q, White and brown adipose tissue. n and o, Control. p, H6PDH. q, 11β-HSD1. r–u, Adrenal gland. r, control. s and t, H6PDH. t, Cortex with medulla inset H6PDH. u, 11β-HSD1. ZF, Zonas fasciculata; ZG, zonas glomerulosa; ZR, zonas reticularis.

Figure 4, e–m, is representative photomicrographs of ihc staining for H6PDH and 11β-HSD1 pairs in adjacent sections of the same kidney. The distribution of immunoreactivity for the two enzymes was quite different. Light to moderate H6PDH immunoreactivity (Fig. 4, e, g, j, and l) was present in most cells of the kidney, except those of the papillary interstitium, and was most intense in epithelial cells of convoluted proximal tubular cells in the juxtamedullary cortex, with only a few convoluted and straight descending proximal tubules of superficial nephrons staining darkly. Staining within these cells was granular, with the highest concentration in the brush border. Podocytes in the glomeruli were

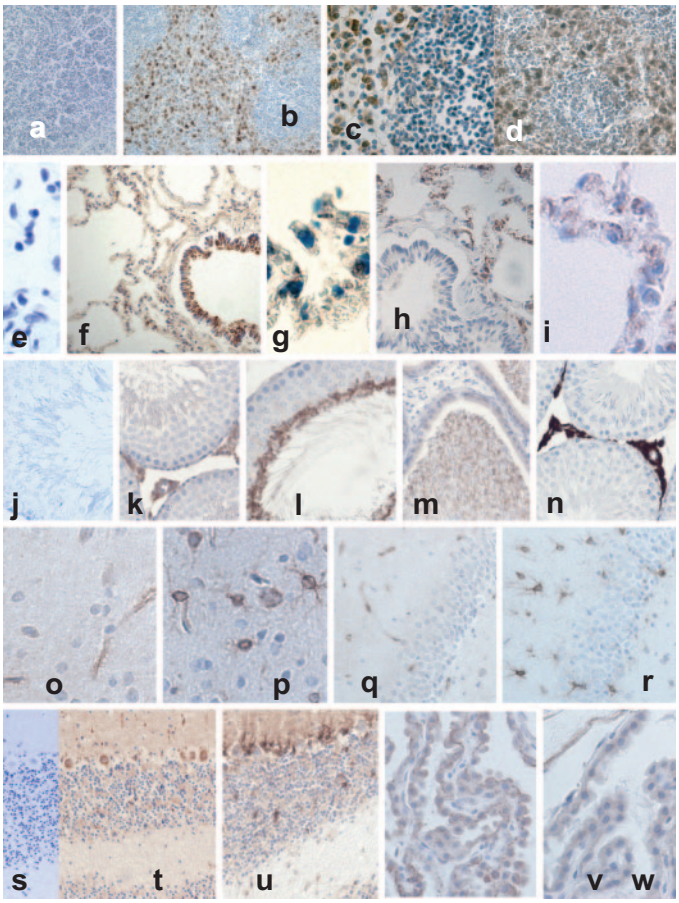

FIG. 5. Photomicrographs of rat tissues using antibodies against the H6PDH and 11β-HSD1 for ihc. a–d, Spleen and lymph node. a, Control spleen. b, Spleen H6PDH. c, Lymph node H6PDH. d, Spleen 11β-HSD1. e–i, Lung. e, Control. f and g, H6PDH. h and i, 11β-HSD1. j–n, Testes and vas deferens. j, Control. k, Seminiferous tubules and Leydig cells H6PDH. l, Seminiferous tubules and sperm H6PDH. m, Epididymis H6PDH. n, Seminiferous tubules and Leydig cells, 11β-HSD1. o–w, Brain: o, Frontal cortex H6PDH. p, Frontal cortex-11β-HSD1. q, Hippocampus H6PDH. r, Hippocampus-11β-HSD. s, Control, cerebellum. t, Cerebellum H6PDH. u, Cerebellum 11β-HSD1. v, Choroid plexus H6PDH. w, Choroid plexus 11β-HSD1.

moderately stained by the H6PDH antibody. Macula densa cells were not darkly stained, but the epithelial cells in inner cortical nephrons across the tubule from the macula densa often were. Staining was less intense in cells of the descending loop of Henle, connecting tubes, and collecting ducts than in those of the proximal tubules. Staining of collecting duct epithelia was uneven, with one to two cells seen on a transverse cut being markedly more stained than the others (Fig. 4l). Epithelial cells of the calyx were also quite immunoreactive to the H6PDH antibodies (data not shown).

11β-HSD1 immunoreactivity in the kidney (Fig. 4, f, h, k, and m) was present in almost all proximal tubules of the superficial cortical nephrons and most of the distal convoluted tubules. 11β-HSD1 immunoreactivity of proximal tubular epithelial cells was of variable intensity within the same cross-section of a tubule but was particularly intense in the brush borders of those cells that were immunoreactive. Podocytes of the glomeruli were lightly stained, as were the macula densa cells. Collecting duct epithelial cells were completely negative, however, most, if not all medullary interstitial cells were very intensely stained (Fig. 4m).

Figure 4, n–q, is of interscapular white and brown adipose tissue; white and brown fat are controls (Fig. 4, n and o). The cytoplasm of white and brown adipocytes were intensely and similarly stained by both the H6PDH (Fig.4p) and 11β-HSD1 (Fig. 4q) antibodies. The cell that was highly immunoreactive for both enzymes may be a fibrocyte, macrophage, or plasma cell because the latter are interspersed throughout adipose tissue and are detected by antibodies against both enzymes, as can be appreciated in the spleen and lymph nodes (Fig. 5, a–d).

Figure 4, r–u, is of the adrenal gland. The H6PDH antibody produced a moderate granular straining of the cytoplasm of adrenal cortical cells (Fig. 4, s and t), with the zona glomerulosa cells staining least intensely, whereas the chromaffin cells were much more intensely stained (*inset*). There was moderate 11β-HSD1 staining of the cells of the adrenal cortex, but not chromaffin cells (Fig. 4u). There was very intense 11β-HSD1 immunoreactivity outside of the chromaffin and zona glomerulosa cells with occasional spots or short streaks radiating through the zona fasciculata/reticularis that may be neuronal processes or fibrocytes.

The pattern of staining in the spleen, lymph nodes (Fig. 5, a–d), and thymus (data not shown) was very similar for both H6PDH and 11β-HSD1 antibodies. Plasma cells of the red pulp were intensely stained, as were macrophages in nonlymphoid tissues. Cells forming the stroma, trabeculae, and capsule were less immunoreactive. The lymphoid cells of the periarteriolar lymphoid sheaths and germinal centers of the white pulp were not stained. Similarly, thymocytes in the germinal centers of the thymus were not appreciably stained, whereas larger cells were.

Photomicrographs of the lung are seen in Fig. 5, e–i, Fig. 5, f and g, being representative of H6PDH staining, and Fig. 5, h and i, of 11β-HSD1 staining. Airway epithelial cells and alveolar and interstitial macrophages were intensely immunoreactive, whereas alveolar cells and type 1 pneumocytes were not stained appreciably with the H6PDH antibody. 11β-HSD1 staining was also seen in alveolar interstitial cells and macrophages, but not in bronchiolar epithelia.

Figure 5, k–n, is representative ihc of the testes and vas deferens. Leydig cells were intensely immunoreactive for H6PDH (Fig. 5k) and 11β-HSD1 (Fig. 5n), whereas the germinal cells were not. However, the maturing and mature spermatozoa, including their tails, were stained by the antibody against H6PDH (Fig. 5, k–m), but not that against 11β-HSD1 (Fig. 5n). The distributions of H6PDH and 11β-HSD1 immunoreactivities in the rat ovary (data not shown) were similar, with the most intense staining in corporal lutea and theca cells, and light or no staining in the granulosa cells and oocytes. Uterine epithelia cells were intensely stained by the H6PDH antibody; that of the endometrial glands slightly less so. Elastic fibers of the myometrium, like those of the aorta, and muscularis layers of the stomach, duodenum, and colon were lightly and nonspecifically immunoreactive.

Figure 5, o–w, is representative micrographs of the cerebral cortex, dentate area of the hippocampus, cerebellum, and choroid plexus of the dorsal third ventricle. Some neurons and the epithelial cells of the pia and choroid plexus

stained with antibodies against both enzymes. Although similar in some areas and cells, *e.g.* magnocellular neurons and large neurons of the nuclei of the seventh cranial nerve (data not shown), the immunoreactivity for H6PDH antibodies clearly differed from that of 11β-HSD1 in others. Most notably, the cytoplasm of the Purkinje cells bodies was stained intensely by the H6PDH antibody (Fig. 5t), but not by the 11β-HSD1 antibody (Fig. 5u), which stained the smaller cells adjacent to the Purkinje cells more strongly.

In the heart (data not shown), there was light ihc staining in a Z-band-like pattern, and clear darker staining of fibrocytes and macrophages. Significant H6PDH and 11β-HSD1 staining of the section of the aorta (data not shown) was confined to adherent fat, fibrocytes, and macrophages. There was no granular staining within the vascular smooth muscle, as would be expected for enzymes within the ER, but wavy elastin in vessels and the heart was lightly stained. Similarly, the elastin in the muscularis layers of the stomach and intestines (data not shown) was lightly stained in a wavy pattern. Epithelial cells of the villi and crypts of Lieberkühn of the duodenum and colon were not immunoreactive, but macrophages between the crypts were intensely stained. Uterine epithelia cells were intensely stained by the H6PDH antibody; that of the endometrial glands slightly less so. Light wavy staining in the myometrium appears to be of the elastic fibers, and, like the elastic fibers of the aorta, muscularis layers of the stomach, duodenal and colon, and heart, are probably nonspecific staining.

Discussion

The significance of H6PDH in the production of NADPH for microsomal metabolism of steroids and drugs was recognized over 30 yr ago by Hori and Takahashi (43). The obligate relationship between reductase activity of the 11β-HSD1 enzyme and H6PDH for reductase activity by the 11β-HSD1 was suggested by a study of cortisol reductase deficiency in humans (44) and confirmed by elegant experiments using differentiating adipocytes (45), as well as a H6PDH knockout mouse (14). Although expression of 11β-HSD1 does not change significantly as preadipocytes become adipocytes, there is a very significant increase in H6PDH expression, resulting in a change in the net 11β-HSD activity from dehydrogenase and inactivation of cortisol in preadipocytes, to reductase, production of cortisol from cortisone, in mature adipocytes (45). As in liver microsomes (13), 11β-HSD1 in adipocytes forms a functional unit with H6PDH and glucose-6-phosphate transporter, required for the transport of substrate into the ER, within the ER (46). As expected, both real time RT-PCR and Western blot indicated that H6PDH and 11β-HSD1 were highly expressed in adipose tissue, and staining of attached fat cells served as a consistent positive control for both enzymes in ihc sections from other tissues.

Use of the ratio of mRNA for 11β-HSD1 to H6PDH (Fig. 1C) controls for variations in GAPDH mRNA in the various tissues. A ratio less than or nearly 1, such as seen in fat, spleen, and thymus, would suggest more than enough NADPH generating capacity to drive 11β-HSD1 reductase activity, though NADPH generation in the ER may serve the cofactor requirement for more than this one enzyme (43, 47). A relatively larger 11β-HSD1 to H6PDH mRNA ratio does not necessarily indicate a decrease in net reductase activity of the 11β-HSD1 in a given tissue. Despite the relative excess of mRNA for 11β-HSD1 to H6PDH in the liver, 11β-HSD1 reductase activity is responsible for the conversion of cortisone and 11-dehydrocorticosterone to cortisol and corticosterone in this organ.

When no permeabilization or antigen retrieval methods were used, the most intense H6PDH and 11β-HSD1 immunoreactivity occurred in hepatocytes closest to the central vein, with staining fading centrifugally, a pattern described previously (2, 12, 42). However, use of a retrieval step described for the ihc detection of syntaxin 3 in renal epithelia (48) produced stronger and more homogeneous immunostaining of hepatocytes for both enzymes and eliminated the pattern of darkest staining around central veins.

The immunohistochemical patterns produced by the H6PDH and 11β-HSD1 antibodies in the kidney were similar to those described by others (2, 12) and quite different from each other. The limited coexpression of H6PDH with 11β-HSD1 in some parts of the kidney suggests that in the healthy rat on a standard rodent diet of 0.3% NaCl, a net 11β-HSD1 dehydrogenase activity converting corticosterone to inactive metabolites may predominate, particularly in the renal medullary interstitial cells, in which staining by the 11β-HSD1 antibody was particularly intense, whereas that by the H6PDH antibodies in the renal medulla was limited to light staining of a few tubular epithelial cells. However, because MR expression in the rat kidney resembles that of H6PDH more than that of 11β-HSD1 (6, 37), it is unlikely that 11β-HSD1 dehydrogenase activity protects the MR from binding by corticosterone in the kidney but regulates occupancy of the GR in these cells. Both GR and MR immunoreactivity were described in rabbit renal medullary interstitial cells (49), in which case dehydrogenase activity of 11β-HSD1 would be expected to protect both from excessive activation by circulating levels of glucocorticoids. The various enzymes for prostaglandin synthesis, particularly the cyclooxygenases, are amply and differentially expressed in the different cells comprising the renal medulla, including the medullary interstitial cells (50), and are modulated by glucocorticoids as well as the renin-angiotensin-aldosterone system, therefore subject to regulation by the 11β-HSD1 and 11β-HSD2 enzymes (51–54). Prostaglandin E_2 synthesis by cyclooxygenase 2 in the renal interstitial cell is particularly important in mitigating the effects of antidiuretic hormone during water deprivation. The extent that 11β-HSD1 activity modulates activation of the corticoid receptors in the renal medulla and prostaglandin generation is a topic for consideration for future studies (55). Renal medullary interstitial cells have been the source of medullipin I, the precursor of medullipin II, and angiolysin, both putative potent vasodepressor agents (56–58). Whether these agents are modulated by glucocorticoids, therefore by 11β-HSD1, is not known.

Limitations of the ihc methods in a previous study allowed detection of H6PDH only in airway epithelium of the lung (12). We have also demonstrated in alveolar macrophages and interstitial fibrocytes, along with 11β-HSD1. The staining in the macrophages and plasma cells but not monocytes by both H6PDH and 11β-HSD1 in ihc of the spleen and

thymus is concordant with the demonstration that 11β-HSD1 is induced in human monocytes upon differentiation to macrophages (59) and suggests that reductase activity of the 11β-HSD1 may be important in modulating the regulation of these cells by glucocorticoids. Similarly, cortisol and corticosterone suppress testosterone synthesis by Leydig cells, and our demonstration of expression of both H6PDH and 11β-HSD1 supports the suggestion that 11β-HSD1 modulates glucocorticoid levels in these cells (60–62).

Our antibody was able to demonstrate H6PDH immunoreactivity in chromaffin cells, which could not be ascertained in a previous study due to nonspecific staining by the secondary antibody (12). In our experience the RAH113 antibody against 11β-HSD1 consistently produced light staining in all adrenal cortical cells, as was reported in ihc of the human adrenal using a different antibody (42). The discrepancy between our results in the adrenal gland and those of others using the same antibody (2) may be due to our use of SDS to enhance antigen retrieval, as well as STF, a noncross-linking fixative, instead of paraformaldehyde and glutaraldehyde.

This study did not support our hypothesis that 11β-HSD1 might act in normal heart and vessels as a dehydrogenase due to the absence of H6PDH. Despite abundant evidence that the heart exhibits significant NADP-dependent 11-dehydrocorticosterone reductase activity (63, 64), expression of both enzymes as assessed by Western blot analysis of heart microsomal protein was low, consistent with previous reports (2). The specificity of the light H6PDH staining within the cardiomyocyte by ihc is suspect because two antibodies against different nonoverlapping portions of the H6PDH molecule detected bands of the same smaller molecular masses (Fig. 3A). Whether these bands were a degradation product, splice variant of the H6PDH, or active posttranslationally cleaved protein is not certain. A recent report of a H6PDH null mouse does not mention a change in cardiovascular phenotype (14). Fibroblasts and macrophages in the heart were immunoreactive in this study and that of Brereton *et al*. (2) and increase greatly in injury. The MR and GR are suspected of interacting at the molecular level, as well as indirectly through synergistic and opposing functions (64, 65), therefore, a change in the rate of generation of corticosterone from circulating 11-dehydrocorticosterone could alter glucocorticoid activation of either or both the MR and GR, and impinge upon homeostasis. The MR in the heart is thought to be inappropriately activated by aldosterone during failure, and it has been reported that 11β-HSD1, but not 11β-HSD2, along with a concomitant increase in the conversion of cortisone to cortisol, is increased in vascular smooth muscle by inflammation (66). Our studies did not address H6PDH levels in the heart or vessels under pathological conditions, however, massive inflammatory cell infiltration occurs early in myocardial infarction and models in which the renin-angiotensin-aldosterone system is increased, and the inflammation and necrosis are mitigated by MR antagonists (67–69).

Studies of the 11β-HSD1 knockout mouse confirmed that the 11β-HSD oxoreductase activity in the brain (70) is due to 11β-HSD1 (71). Low levels of 11β-HSD1 and H6PDH in the whole brain RNA and protein samples are a reflection of their selective expression in specific cells. H6PDH immunoreactivity by ihc in the brain was clearly limited to a small number of neurons in a pattern similar to that of the 11β-HSD1 ihc in some areas, but clearly different in others. Studies demonstrating cellular colocalization, or the lack thereof, are required to determine expression in the same neurons before one can propose that dehydrogenase activity by 11β-HSD1 protects the MR from glucocorticoid occupation, allowing aldosterone to activate the receptor.

Aldosterone modulates cerebrospinal fluid production, presumably through the MR of choroid plexus epithelia (72, 73). We were intrigued by the possibility that hydroxysteroid dehydrogenase activity of 1β-HSD1 might protect the MR of the choroid plexus because we had demonstrated intense MR expression in these cells (37), as well as ihc staining for the α, β, and γ-subunits of the epithelial sodium channel, but not for 11β-HSD2 (unpublished data). Because both 11β-HSD1 and H6PDH are clearly expressed in the choroid plexus, it is unlikely that this is a mechanism conferring aldosterone selectivity to the MR in choroid plexus epithelium. However, in addition to regulating levels of active glucocorticoid, 11β-HSD1 also modulates the equilibrium of 7-keto- and 7-hydroxy-neurosteroids, an activity that also requires coexpression of 11β-HSD1 with H6PDH (47).

In conclusion, we have disproved our hypothesis that hydroxysteroid dehydrogenase activity of 11β-HSD1 expressed without H6PDH confers extrinsic selectivity of the MR for aldosterone in the normal heart or choroid plexus; neither enzyme is expressed in significant amounts in normal cardiomyocyte and vascular smooth muscle cells, and both are expressed in large amounts in the choroid plexus epithelia. 11β-HSD1 was coexpressed with H6PDH in the tissues examined with the notable exception of kidney medullary interstitial cells, in which 11β-HSD1 was expressed without significant H6PDH. Further studies using concomitant labeling techniques are necessary to determine if the two enzymes are expressed in the same neurons with the MR. Although our interest in the H6PDH enzyme was as the determinant of the enzymatic activity of 11β-HSD1 and its possible effects on the modulation of local concentrations of active glucocorticoid, H6PDH activity is crucial to many other ER-based enzymatic activities. The antibody against H6PDH described here should be useful for the study of the role of H6PDH in a wide variety of metabolic functions.

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