乳腺および腎臓における
グルココルチコイド代謝の比較生理学的研究

（研究課題番号　07806037）

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はしがき

本報告書は平成7、8年度の2年間にわたる科学研究費補助金（基盤研究C）『乳腺および腎臓におけるグルココルチコイド代謝の比較生理学的研究』の研究成果をまとめたものである。

腎臓のようなミネラロコルチコイドの標的器官には、グルココルチコイドの11位水酸基をケトン基に変換するステロイド11β水酸基脱水素酵素が存在する。この酵素の存在は、逆に、ミネラロコルチコイドの標的器官であることの証明につながると考え、本研究では乳腺、腎臓、および卵管のグルココルチコイド代謝を、マウスとウズラを用いて比較検討した。

高等動物の電解質代謝および水の排泄・分泌は非常に複雑に調節されている現象であるが、本研究ではこれを分子レベルで解明することによって、少しでも理解を深めようと努力した。

本報告書が内分泌学を研究するものにとって参考になれば幸いである。

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研究発表

（1）学会誌等

Mady, M. E. I., M. Ikami, T. Sasanami and M. Mori： Effect of thyroid stimulating hormone and thyroid hormone on progesterone sysnthesis in cultured granulosa cells of Japnese quail. Japnese Poultry Science, Vol. 34, in press, 1997 年

Kuroki, M. and M. Mori： Effect of protease inhibitor on penetration and binding of spermatozoa to perivitelline layer of quail ovum. Poultry Science, Vol. 76, in press,.1997 年

森　誠, 黒木 雅代： 精子によって形成されるウズラ卵黄膜内層の孔について. 日本畜産学会報, 68 巻 1 号, 54-60 頁, 1997 年 1 月

Mori, M. and M. Kuroki： Specific binding of spermatozoa to the inner layer of vitelline membrane over the germinal disc region of quail eggs. Proceeding of 8th AAAP Animal Science Congress, Vol. 2, 518-519, 1996 年 10 月

Mori, M., T. Sasanami and M. Kuroki： Production of progesterone and inner vitelline membrane protein in quail granulosa cells in culture. Proceeding of 10th International Congress of Endocrinology, Vol. 2, 835, 1996 年 6 月

Kuroki, M. and M. Mori： Origin of 33kDa protein of vitelline membrane of quail egg: Immunological studies. Development Growth & Differentiation, Vol. 37, No 5, 545-550, 1995 年 11 月

浅井 辰夫, 青島 有美, 森　誠, 土井 廣, 中田 史雄： コーヒーカスを添加して堆肥化した牛糞の肥料価値. 畜産の研究, 49 巻 6 号, 704-706 頁, 1995 年 6 月

青島 有美, 森　誠, 浅井 辰夫, 土井 廣, 中田 史雄： 牛糞の堆肥化に及ぼすコーヒーカスの効果. 東海畜産学会報, 5 巻 1 号, 60-62 頁, 1995 年 3 月

（2）口頭発表

笹浪 知宏, 森　誠： ウズラ顆粒膜細胞の増殖と分化におけるシグナル伝達経路. 日本家禽学会春季大会, 1997 年 3 月

森　誠, 前田 ふみ, 酒井 仙吉： ウズラ卵管のステロイド 11 β水酸基脱水素酵素について. 第 92 回日本畜産学会大会, 1997 年 3 月

森　誠, 黒木 雅代: ウズラ卵黄膜と精子の結合に対する抗 33kDa 抗体の影響. 日本家禽学会秋季大会, 1996 年 11 月

笹浪 知宏, 森　誠： ウズラ顆粒膜細胞の増殖と卵黄膜タンパクおよびプロゲステロン合成の関係について. 第 21 回鳥類内分泌研究会, 1996 年 11 月

黒木 雅代, 森　誠: ウズラの卵黄膜と精子の結合について. 日本家禽学会春季大会, 1996 年 3 月

伊神 真紀, 笹浪 知宏, 森　誠, M. E. I. Mady： ウズラ顆粒膜細胞のプロゲステロン合成に対する甲状腺ホルモンの効果. 日本家禽学会春季大会, 1996 年 3 月

笹浪 知宏, 森　誠： ウズラ卵胞顆粒膜細胞の卵黄膜タンパク合成におよぼす各種ホルモンの効果. 第 20 回鳥類内分泌研究会, 1995 年 11 月

笹浪 知宏, 黒木 雅代, 森　誠： ウズラ卵胞顆粒膜細胞の増殖および卵黄膜タンパクとプロゲステロン合成について. 日本比較内分泌学会, 1995 年10 月

森　誠： 家禽の卵黄膜由来の細胞接着因子の探索. 信州農林科学振興会, 研究報告講演会, 1995 年 9 月

（3）出版物

水野 丈夫, 北原 隆, 森　誠, 百瀬 忠征, 野中 繁, 小林 秀明： 生物の世界 1A（文部省検定済高等学校理科用教科書） 東京書籍, 1997 年 3 月

Abstract Corticosteroid action is modulated by the NAD^+-dependent 11β-HSD-2, which oxidatively inactivates corticosterone, allowing the nonselective mineralocorticoid receptor to be occupied by mineralocorticoids. The present study was conducted to show that 11β-HSD-2 is present in the reproductive tract of egg-laying birds.

The microsome fraction was prepared from homogenates of various parts of oviduct of the Japanese quail, and incubated with [^{3}H]corticosterone for 1 hr at 39 C in the presence or absence of 1 mM NAD^+ or $NADP^+$. The reaction was stopped, and the steroid was extracted by ethyl acetate. Separation of steroids was performed using a thin-layer chromatography developed in a chloroform-methanol (95:5) system. Activity of 11β-HSD-2 was determined by measuring the conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone.

As the results, the shell gland and albumen secreting region of the oviduct showed high activity of 11β-HSD-2. In these tissue, [^{3}H]corticosterone was exclusively metabolized to [^{3}H]11-dehydrocorticosterone. On the other hands, infundibulum and isthmus of the oviduct and the vagina contain low activity, indicating that these parts might not be a target of the mineralocorticoids.

Key words: 11β-Hydroxysteroid dehydrogenase, Oviduct, Japanese quail, Corticosterone, Aldosterone

Corticosteroid action is modulated by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) (Edwards *et al.*, 1988; Funder *et al.*, 1988). Two isozymes of 11β-HSD are now recognized: 11β-HSD-1 is NADPH/$NADP^+$-dependent enzyme with a Km in the micromolar range (Monder and White, 1993; Funder, 1993; Seckl, 1993). 11β-HSD-2 is NAD^+-dependent enzyme with a Km in the nanomolar range. In the liver, 11β-HSD-1 is predominant and converts 11-dehydrocorticosterone to corticosterone (Lakshmi and Monder, 1988). On the other hand, in the kidney, the NAD^+-dependent 11β-HSD-2 oxidatively inactivates corticosterone (Rusvai and Naray-Fejes-Tosh, 1993), allowing the nonselective mineralocorticoid receptor (Krozowski and Funder, 1983) to be occupied by mineralocorticoids (Albiston *et al.*, 1994; Krozowski *et al.*, 1994; Rusvai and Naray-Fejes-Tosh, 1993).

The present study was conducted to show that 11β-HSD-2 is present in other sodium-transporting epithelia such as those in the mammary gland of mammals and in the reproductive tract of egg-laying birds.

Materials and Methods

Animals:

Japanese quail were obtained from Tokai-Yuki at 6 wk of age. Individually caged birds were maintained under a lighting schedule of 14 h light (0500 to 1900 h) and 10 h dark

and provided *ad libitum* access to water and a commercial quail diet.

Tissue preparation:

The reproductive tract and kidney of Japanese quail were isolated immediately after decapitation, finely chopped with scissors and washed several times with an ice-cold saline. The tissue was homogenized in 5 vol of homogenizing buffer (0.25 M sucrose, 50 mM Tris-HCl and 5 mM magnesium chloride, pH 7.4) with a Polytron-type homogenizer for 10 sec. The microsome fraction was prepared from homogenates by centrifugation at 10,000 x *g* for 20 min at 4 C, followed by a high speed centrifugation of the supernatant at 105,000 x *g* for 60 min at 4 C. Microsomes were resuspended in the homogenizing buffer.

Enzymatic analysis:

11β-HSD-2 activity in microsome fraction was determined by measuring the conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone. [1, 2, 6, 7-^{3}H]Corticosterone (spec. act., 3.22 TBq/mmol) was purchased from Amersham International plc, England. About 12.3 kBq of ^{3}H-labeled corticosterone dissolved in ethanol was transferred into an incubation tube. After addition of 50 μl of propylene glycol, the solvent was evaporated under reduced pressure immediately before incubation. An appropriate amount of microsome fraction, whose protein content had been previously measured, was added to each tube. NAD^+ and $NADP^+$ was dissolved in the homogenizing

buffer and added to the tube so that the final concentration of the cofactor was 1 mM. Then the final volume of the incubation mixture was adjusted to 0.5 ml per tube by the homogenizing buffer. The above mixture was incubated at 39 C in a Dubnoff-type incubator under aerobic condition. For control experiments the mixture excluding the microsome fraction was incubated simultaneously under the identical condition.

Separation of the metabolites:

At the end of incubation, 1 ml of ethyl acetate was added to the incubation mixture to terminate further enzymatic reaction. The ethyl acetate layer was separated and reserved as an extract. The residual aqueous layer was extracted five-times more to complete extraction of the steroids. The extracts were pooled and an aliquot of the extract was spotted on a thin layer of silica gel GF (E. Merck, Darmstadt, Germany), together with corticosterone and 11-dehydrocorticosterone as markers. After the development with chloroform: methanol (95:5) system, the markers on the chromatogram were detected under an ultraviolet lamp (wavelength, 253 nm). Radioactive areas were detected on another chromatogram by the fluorographic technique. The radioactive area were scraped off from the plates, and the steroids were eluted from the absorbent with chloroform: ethanol (1:1) mixture.

Quantitation of the metabolites, protein and enzyme activity:

Radioacitivities were measured by a liquid scintillation spectrometer, whose counting efficiency of tritium was about 50 %. Protein contents in microsome fraction was measured by the copper-Folin method. The enzyme activities were tentatively expressed as amount in pmole of 11-dehydrocorticosterone produced for 1 hr per mg of microsome fraction.

Results and Discussion

As shown in Fig. 1, 11-dehydrocorticosterone was obtained as the major metabolite of corticosterone after the incubation with microsome fraction of the kidney. No metabolites were detected in the absence of cofactor. Corticosterone was also metabolized to 11-dehydrocorticosterone by the microsome fraction of the whole oviduct.

The regional distribution of the enzyme was shown in Fig.2. Highest activity was observed in the shell gland in the incubation with NAD as the cofactor. The albumen secreting region of the oviduct also showed high activity of 11β-HSD-2. On the other hands, infundibulum and isthmus of the oviduct and the vagina contain low activity, indicating that these parts might not be a target of the mineralocorticoids.

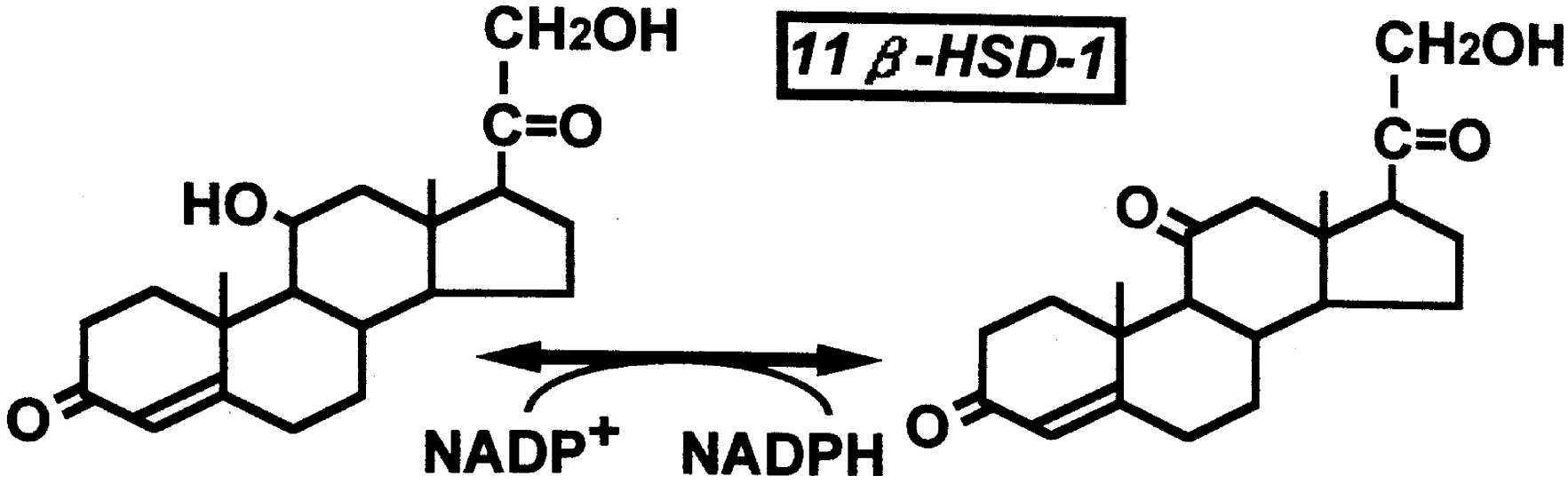
The 11β-HSD-2 enzyme converts glucocorticoids to receptor-inactive metabolites, endowing specificity on the mineralocorticoid receptor and modulating glucocorticoid

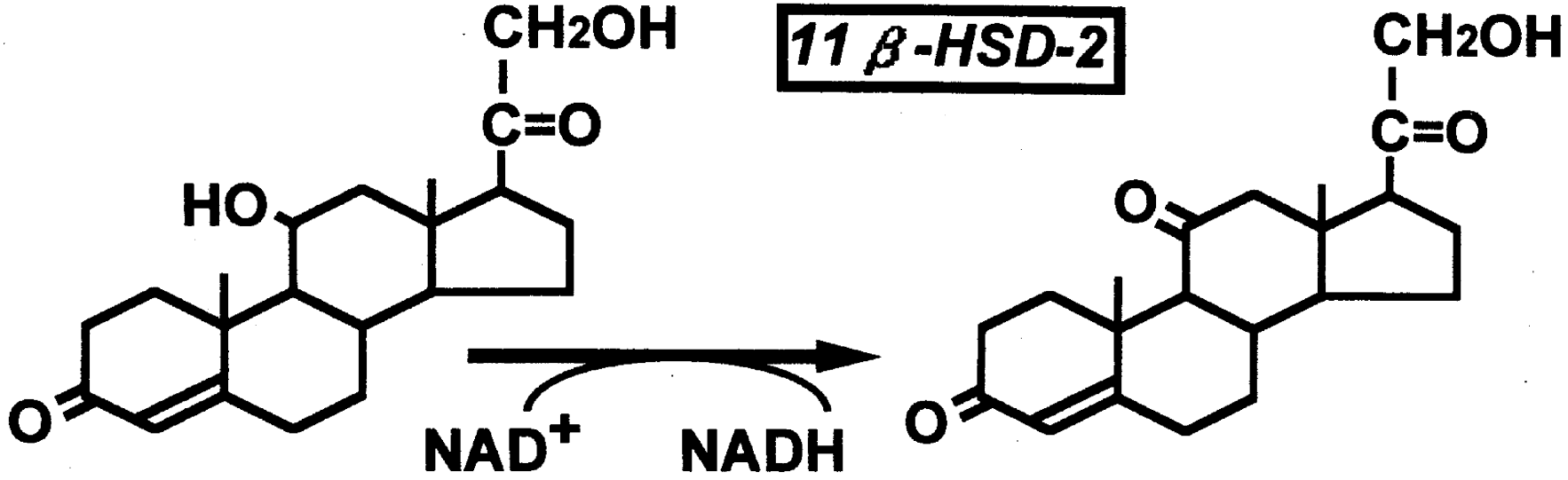
access to the glucocorticoid receptor. The 11β-HSD-2 enzyme has previously been localized in high amounts in classical sodium-transporting epithelia, the ileum and term placenta in humans (Smith *et al.*, 1996; Krozowski *et al.*, 1995; Kyossev *et al.*, 1996; Brown *et al.*, 1996), but studies in the rat have been limited to the detection of message (Whorwood *et al.*, 1995; Roland *et al.*, 1995).

Developmental studies showed the presence of low numbers of 11β-HSD-2-positive tubules in the fetal rat kidney before birth. Studies in other species have provided evidence for fetal 11β-HSD-2 activity, with highest amounts in the kidney (Stewart *et al.*, 1994), whereas in the mouse, renal 11β-HSD-2 mRNA is present by 13 days gestation, preceding the appearance of message of mineralocorticoid receptor (Brown *et al.*, 1996).

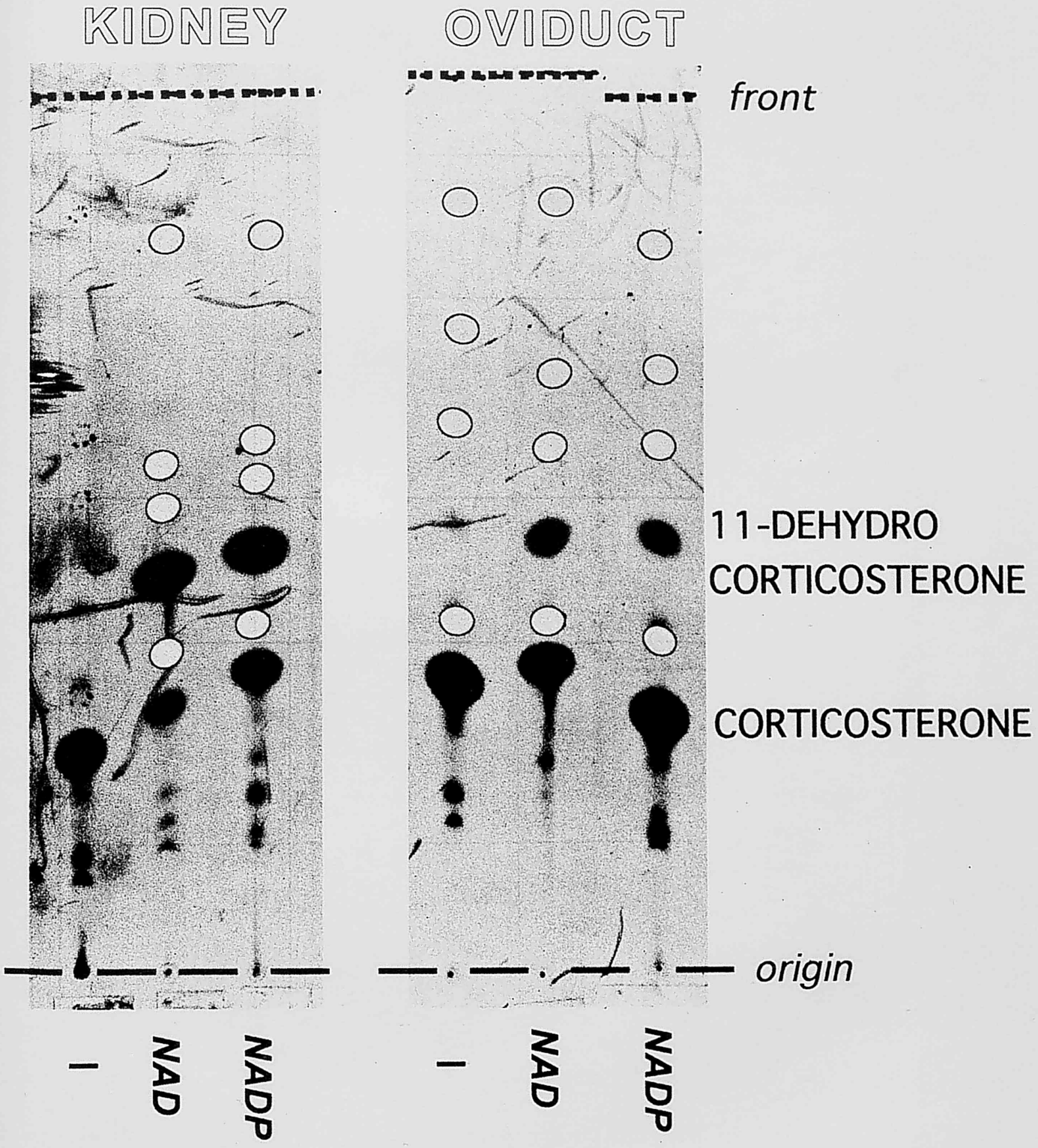
The bile duct and gall bladder absorb water to concentrate bile fluid (Tavoloni, 1985), but this process is not known to be mediated by mineralocorticoids.

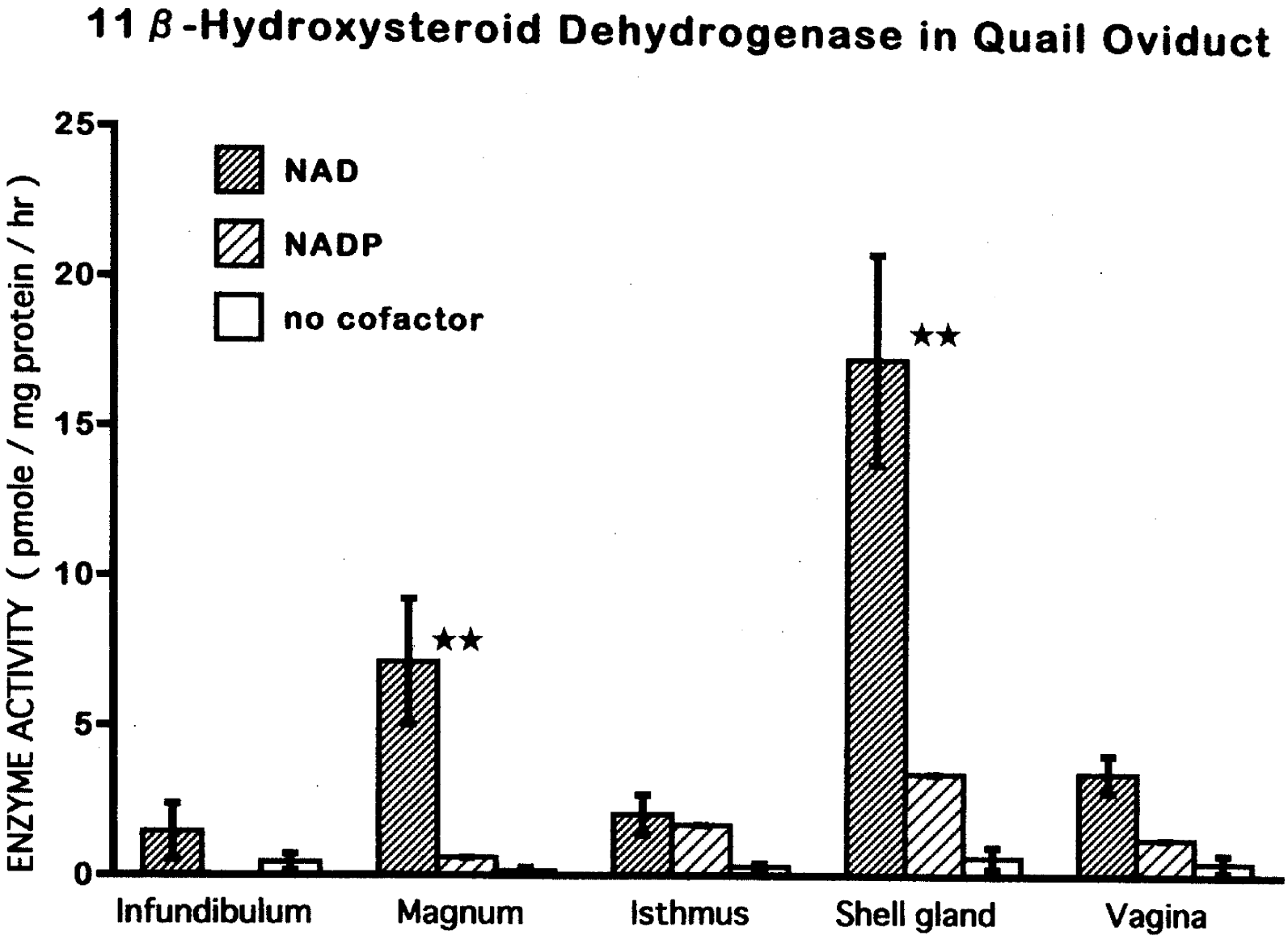
Currently, little is known about the modulation of 11β-HSD-2 activity, although recent studies suggest that it could be regulated by the protein kinase A pathway (Pasquarette *et al.*, 1996) and via corticosteroids (Li *et al.*, 1996).


11 β-HSD-1
CH2OH
C=O
HO
O
NADP+
NADPH
CH2OH
C=O
O
O


11 β-HSD-2
CH2OH
C=O
HO
O
NAD+
NADH
CH2OH
C=O
O
O

Fluorography of TLC




11 β-Hydroxysteroid Dehydrogenase in Quail Oviduct
ENZYME ACTIVITY (pmole / mg protein / hr)
25
20
15
10
5
0
NAD
NADP
no cofactor
★★
★★
Infundibulum
Magnum
Isthmus
Shell gland
Vagina

References

Agarwal AK, Mune T, Monder C, White PC. NAD(+)-dependent isoform of 11β-hydroxysteroid dehydrogenase: cloning and characterization of cDNA from sheep kidney. J. Biol. Chem., 269:25959-25962. 1994.

Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS. Cloning and tissue distribution of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme. Mol. Cell. Endocrinol., 105:R11-R17. 1994.

Belanger B, Fiet J, Belanger A. Effects of adrenocorticotropin on adrenal and plasma 11β-hydroxyandrostenedione in the guinea pig and determination of its relative androgen potency. Steroids, 58:29-34. 1993.

Brown RW, Chapman KE, Kotelevtsev Y, Yau JLW, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CRW, Seckl JR. Cloning and production of antisera to human placental 11β-hydroxysteroid dehydrogenase type 2. Biochem. J., 313:1007-1017. 1996.

Brown RW, Diaz R, Robson AC, Kotelevtsev YV, Mullins JJ, Kaufman MH, Seckl JR. The ontogeny of 11β-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene

expression reveal intricate control of glucocorticoid action in development. Endocrinology, 137:794-797. 1996.

Cole TJ. Cloning of the mouse 11β-hydroxysteroid dehydrogenase type 2 gene: tissue specific expression and localization in distal convoluted tubules and collecting ducts of the kidney. Endocrinology, 136:4693-4696. 1995.

Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, deKloet ER, Monder C. Localization of 11β-hydroxysteroid dehydrogenase - tissue specific protector of the mineralocorticoid receptor. Lancet, 2:986-989. 1988.

Farman N, Oblin ME, Lombes M, Delahaye F, Westphal HM, Bonvalet JP, Gasc JM. Immunolocalization of gluco- and mineralocorticoid receptors in rabbit kidney. Am. J. Physiol., 260:C226-C233. 1991.

Funder JW, Pearce PT, Smith R, Smith AI Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science, 242:583-585. 1988.

Heffernan M, Dennis JV. Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells. Nucleic Acids Res., 19:85-92. 1991.

Henning SJ. Postnatal development: coordination of feeding, digestion, and metabolism. Am. J. Physiol. 241:G199-G214. 1981.

Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat. Genet., 12:325-328. 1996.

Jackson BA, Braun-Wemess JL, Kusano E, Dousa TP. Concentrating defect in the adrenalectomized rat. Abnormal vasopressin-sensitive cyclic adenosine monophosphate metabolism in the papillary collecting duct. J. Clin. Invest. 72:997-1004. 1983.

Jenkins JS. The metabolism of cortisol by human extrahepatic tissues. J. Endocrinol., 34:51-56. 1965.

Kenouch S, Alfaidy N, Bonvalet JP, Fannan N. Expression of 11β-HSD along the nephron of mammals and humans. Steroids, 59:100-104. 1994.

Krozowski Z, Stuchbery S, White P, Monder C, Funder JW. Characterization of 11β-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. Endocrinology., 127:3009-3013. 1990.

Krozowski ZS, Funder JW. Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. Proc. Natl. Acad. Sci. USA, 80:6056-6060. 1983.

Krozowski Z, Maguire JA, Stein-Oakley AN, Dowling J, Smith RE, Andrews RK. Immunohistochemical localization of the 11β-hydroxysteroid dehydrogenase type Ⅱ enzyme in human kidney and placenta. J. Clin. Endocrinol. Metab., 80:2203-2200. 1995.

Krozowski Z, Wendell K, Ahima R, Harlan R. Type-Ⅰ corticosteroid receptor-like immunoreactivity in the rat salivary glands and distal colon: modulation by corticosteroids. Mol. Cell. Endocrinol., 85:21-32. 1992.

Kyossev Z, Walker PD, Reeves WB. Immunolocalization of NAD-dependent 11β-hydroxysteroid dehydrogenase in human kidney and colon. Kidney Int., 49:271-281. 1996.

Li KXZ, Smith RE, Ferrari P, Funder JW, Krozowski ZS. Rat 11β-hydroxysteroid dehydrogenase type 2 enzyme is expressed at low levels in the placenta and is modulated by adrenal steroids in the kidney. Mol. Cell. Endocrinol., 120:67-75. 1996.

Mercer WR, Krozowski ZS. Localization of an 11β hydroxysteroid

dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. Endocrinology, 130:540-543. 1992.

Monder C, Lakshmi V. Evidence for kinetically distinct forms of corticosteroid 11β-dehydrogenase in rat liver microsomes. J. Steroid. Biochem., 32:77-83. 1989.

Monder C, Lakshmi V. Corticosteroid 11β-dehydrogenase of rat tissues: Immunological studies. Endocrinology, 126:2435-2443. 1990.

Mune T, Rogerson FM, Nikkila H, Agarwal AK, White PC. Human hypertension caused by mutations in the kidney isozyme of 11β-hydroxysteroid dehydrogenase. Nat. Genet., 10:394-399. 1995.

NarayFejesToth A, FejesToth G. Subcellular localization of the type 2 11β-hydroxysteroid dehydrogenase: a green fluorescent protein study. J. Biol. Chem., 271:15436-15442. 1996.

Page N, Warriar N, Govindan MV. 11β-Hydroxysteroid dehydrogenase and tissue specificity of androgen action in human prostate cancer cell LNCaP. J. Steroid Biochem. Mol. Biol., 49:173-181. 1994.

Pasquarette MM, Stewart PM, Ricketts ML, Imaishi K, Mason JI.

Regulation of 11β-hydroxysteroid dehydrogenase type 2 activity and mRNA in human choriocarcinoma cells. J. Mol. Endocrinol., 16:269-275. 1996.

Perschel FH, Buhler H, Hierholzer K. Bile acids and their amidates inhibit 11β-hydroxysteroid dehydrogenase obtained from rat kidney. Pflugers Arch. 418:538-543. 1991.

Roland BL, Li K, Funder JW. Hybridization histochemical localization of 11β-hydroxysteroid dehydrogenase type 2 in rat brain. Endocrinology, 136:4697-4700. 1995.

Roland BL, Funder JW. Localization of 11β-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. Endocrinology, 137:1123-1128. 1996.

Roland BL, Krozowski ZS, Funder JW. Glucocorticoid receptor, mineralocorticoid receptors, 11β-hydroxysteroid dehydrogenase-1 and -2 expression in rat brain and kidney: in situ studies. Mol. Cell. Endocrinol., 111:R1-R7. 1995.

Rusvai E, Naray-Fejes-Toth A. A new isoform of 11-β-hydroxysteroid dehydrogenase in aldosterone target cells. J. Biol. Chem., 268:10717-10720. 1993.

Sasano H, Fukushima K, Sasaki I, Matsuno S, Nagura H, Krozowski ZS. Immunolocalization of mineralocorticoid receptor in human kidney, pancreas, salivary, mammary and sweat glands: a light and electron microscopic Immunohistochemical study. J. Endocrinol., 132:305-310. 1992.

Schmid G, Heidland A. Cation excretion and kallikrein secretion by the rat parotid gland in various hypertensive models. J. Cardiovasc. Pharmacol., 6:S101-S106. 1984.

Sheppard KE, Funder JW. Specific nuclear localization of 11-dehydrocorticosterone in the rat colon: evidence for a novel corticosteroid receptor. Endocrinology, 137:3274-3278. 1996.

Stewart PM, Murry BA, Mason JI. Type 2 11β-hydroxysteroid dehydrogenase in human fetal tissues. J. Clin. Endocrinol. Metab., 78:1529-1532. 1994.

Smith RE, Maguire JA, Stein-Oakley AN, Sasano H, Takahashi K, Fukushima K, Krozowski ZS. Localization of 11β-hydroxysteroid dehydrogenase type Ⅱ in human epithelial tissues. J. Clin. Endocrinol. Metab. 81:3244-3248. 1996.

Stewart PM, Krozowski ZS, Gupta A, Milford DV, Howie AJ, Sheppard MC, Whorwood CB. Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11β-

hydroxysteroid dehydrogenase type 2 gene. Lancet, 347:88-91. 1996.

Stewart PM, Whorwood CB, Barber P, Gregory J, Monder C, Franklyn JA Sheppard MC. Localization of renal 11β-dehydrogenase by in situ hybridization: autocrine not paracrine protector of the mineralocorticoid receptor. Endocrinology, 128:2129-2135. 1991.

Tavoloni N. Role of ductular bile water reabsorption in canine bile secretion. J. Lab. Clin. Med., 106:154-161. 1985.

Ullian ME, Walsh LG. Corticosterone metabolism and effects on angiotensin II receptors in vascular smooth muscle. Circ. Res., 77:702-709. 1995.

Whorwood CB, Ricketts ML, Stewart PM. Epithelial cell localization of type 2 11β-hydroxysteroid dehydrogenase in rat and human colon. Endocrinology, 135:2532-2541. 1994.

Whorwood CB, Mason JI, Ricketts ML, Howie AJ, Stewart PM. Detection of human 11β-hydroxysteroid dehydrogenase isoforms using reverse-transcriptase-polymerase chain reaction and localization of the type 2 isoform to renal collecting ducts. Mol. Cell. Endocrinol., 110:R7-R12. 1995.

Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghyazar M, Harbison MD, Wei JQ, Shackleton C, Funder JW, New MI. A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. J. Clin. Endocrinol. Metab., 80:2263-2266. 1995.

Work J, Jamison RL. Effect of adrenalectomy on transport in the rat medullary thick ascending limb. J. Clin. Invest., 80:1160-1164. 1987.

Yang K, Matthews SG. Cellular localization of 11β-hydroxysteroid dehydrogenase 2 gene expression in the ovine adrenal gland. Mol. Cell. Endocrinol., 111:R19-R23. 1995.

Zhou MY, Gomez-Sanchez EP, Cox DL, Cosby D, Gomez-Sanchez CE. Cloning expression, and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent 11β-hydroxysteroid dehydrogenase. Endocrinology, 136:3729-3734. 1995.