Glucocorticoid Metabolism in the Newborn Rat Heart (43888)

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Abstract. **A relative cardiac hypertrophy has been observed in newborns chronically treated with dexamethasone. To test the hypothesis—that dexamethasone might alter steroid metabolism within the heart—rat pups were injected with vehicle, corticosterone (dosages 20 or 200 μg/pup/injection, or 1 mg/pup/injection) or dexamethasone (5 μg/pup/injection) on Day 2–6 and sacrificed on Day 7–8. Injections with dexamethasone in this dosage have induced the cardiac changes in this rat model. 11β-Hydroxysteroid dehydrogenase (11β-OHSD) activity was assessed in hearts from these adrenally intact rat pups by incubating tissues with ^{3}H-corticosterone 10^{-8} *M* for 60 min. On Day 7–8, controls transformed 10.3% ± 1.1% (mean ± SE) of the corticosterone (Compound B) to 11-dehydrocorticosterone (Compound A) generating 1.25 ± 0.35 × 10^{-12} moles A/mg protein (n = 8). Tissues from pups pretreated with corticosterone at all three dosages were not different from controls in percent metabolized and moles A/mg generated. In contrast, hearts from dexamethasone treated pups transformed only 4.5% ± 1.0% of the corticosterone to A generating 3.19 ± 0.05 × 10^{-13} moles A/mg protein (n = 10) ($P < 0.05$ versus control in moles/mg protein metabolized). Cultured cardiomyocytes exposed to dexamethasone for 4 days in vitro also decreased their expression of 11β-OHSD mRNA. Readily metabolized endogenous glucocorticoids produced little or no effect on developing heart muscle while treatment with dexamethasone, a potent synthetic glucocorticoid, induced relative cardiac hypertrophy and downregulated 11β-OHSD mRNA expression and enzyme activity.** [P.S.E.B.M. 1995, Vol 209]

The enzyme, 11β-hydroxysteroid dehydrogenase (11β-OHSD) metabolizes circulating endogenous glucocorticoids to their respective "biologically inert" 11-dehydro derivatives i.e. conversion of corticosterone (compound B) to 11-dehydrocorticosterone (compound A) (1–6). The activity of this enzyme in target tissues can directly determine the amount of glucocorticoid within the cell available to bind to either a type I (mineralocorticoid) or type II (glucocorticoid) receptor and initiate a biological response. While the kidney and liver appear to be the sites of greatest enzyme activity (1–4), it is now clear that the heart also possesses a significant amount of 11β-OHSD (7). Since endogenous glucocorticoids and mineralocorticoids bind to the mineralocorticoid receptor with equal affinity in vitro (8) and since type I mineralocorticoid receptors are present in the heart (9), it is quite likely that 11β-OHSD regulates the binding of glucocorticoids to the type I receptor in this organ.

A relative cardiomyopathy characterized by an increase in the thickness of the intraventricular septum and left ventricular free wall has been described in ventilator dependent premature infants chronically treated with the synthetic glucocorticoid, dexamethasone (10) as well as infants given long-term therapy with ACTH (11). Changes in the right ventricular free wall thickness have also been noted but appear less pronounced (10). Similar cardiac findings have been

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produced in the newborn Sprague-Dawley rat model administered parenteral dexamethasone from Day 2 to Day 6 of life (12). Myocardial fibrosis and ventricular hypertrophy have also been noted in animal models of mineralocorticoid excess (13, 14). Adult Sprague-Dawley rats chronically administered either aldosterone or deoxycorticosterone (DOC) exhibit these changes in myocardial structure independent of blood pressure. Since the presence of excess steroid hormones correlates with rather specific myocardial changes, it seems likely that the metabolism of steroid hormones in cardiac myocytes is important in the development of hypertrophy.

The synthetic glucocorticoid, dexamethasone, induces many varied effects on the heart. It promotes the induction of a number of proteins and induces differentiation of myocytes in culture (15, 16). Dexamethasone exposure increases the level of atrial natriuretic peptide mRNA in cardiomyocytes leading directly to an increase in peptide itself (15). Given the findings of relative cardiac hypertrophy in the newborn following dexamethasone exposure, we hypothesized that chronic administration of this synthetic glucocorticoid might directly affect 11β-OHSD activity, possibly at the level of mRNA expression. Our studies, conducted in the newborn Sprague-Dawley rat model, confirm that chronic administration of dexamethasone results in a relative ventricular hypertrophy similar to that seen in the human newborn. Furthermore, we demonstrate that these cardiac changes are associated with a significant decrease in 11β-OHSD enzymatic activity and level of mRNA expression.

Materials and Methods

The ^{3}H-corticosterone was obtained from New England Nuclear (Boston, MA). Nonradioactive steroids were from Sigma Corporation (St. Louis, MO). Pregnant Sprague-Dawley rats were acquired from Charles River breeding laboratories (Wilmington, MA).

Rats were housed individually in plastic cages with free access to food and water. At term, the litters were delivered naturally. The rat pups were allowed to nurse freely until just prior to sacrifice. The rat pups were injected with either vehicle or a test steroid on Day 2 through 6 and sacrificed on Day 7 or 8. The steroids used were either corticosterone (B) at a dose of 20 μg/pup/injection (low B), 200 μg/pup/injection (medium B), or 1 mg/pup/injection (high B), or dexamethasone 5 μg/pup/injection. The dose of dexamethasone chosen has been shown to induce the cardiac changes in this rat model (12). Dexamethasone is approximately 40 times more potent as a glucocorticoid compared with corticosterone on a weight basis.

11β-OHSD activity was assessed in minces of cardiac tissue derived from these adrenally intact rat pups. Following sacrifice, the hearts were removed and placed into iced mammalian Ringer's solution pH 7.4. Tissue minces were prepared using a scalpel blade. The minces then were transferred into vials containing 1 ml of preoxygenated mammalian Ringer's solution at 37°C. Enzyme activity was assessed by the conversion of corticosterone to 11-dehydro corticosterone after a 60-min incubation. At the conclusion of the 60-min incubation, 2 ml of methanol were added to the vial to end the reaction. Samples were centrifuged and the supernatant containing the steroids was evaporated under a stream of nitrogen in preparation for high pressure liquid chromatography (HPLC). The HPLC was conducted using a Dupont Zorbax C8 column eluted at 44°C with a flow rate of 1 ml/min using 45% methanol for 30 min and increasing linearly to 100% methanol over an additional 15 min. The ^{3}H-corticosterone and its metabolites were identified comparing retention times to known standards by monitoring radioactivity on line with a Berthold LB 504 detection system.

In selected experiments, cardiac myocytes were isolated for primary cell culture. Two- to five-day-old rats were placed on ice for 10 min and sacrificed by decapitation. Hearts were dissected, minced, and placed into 10 ml fresh ice-cold Hanks' balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY). The tissue pieces were washed three times in sterile HBSS and transferred to new petrie dishes containing 10 ml collagenase (Sigma, St. Louis, MO) solution (sterile filtered 1 mg/ml HBSS). Cardiac tissues were broken down by repeated passages through a siliconized, sterile pipette 10 times every 15 min for 1 hr, until completely dissolved. Samples were then transferred to 50-ml sterile conical tubes. Ten percent horse serum (pooled donor herd) (Gibco BRL) was added to the dissolved tissues and the samples were centrifuged at 800 rpm for 15 min. The supernatant was removed and cells were centrifuged a second time in 10% horse serum at the same speed for 15 min. The supernatant was discarded and cells resuspended in DMEM (Gibco BRL) with 10% fetal bovine serum (Gibco BRL). Cells were transferred to tissue culture flasks and allowed to adhere for one hour at 37°C. After 1 hr, the cells were removed, transferred to 15-ml conical tubes, and resuspended in DMEM with 10% fetal bovine serum. Cells were initially cultured with ARA-C for 24 hr to prevent the growth of fibroblasts. When prepared by this method, nests of spontaneously beating cells formed and secreted atrial natriuretic peptide (ANP). Following previously published methods (17), the ANP generated has been measured in unextracted media samples diluted 1:3 with DMEM using an enzyme-linked immunoassay (Cayman Chemical Company, Ann Arbor, MI) and an unextracted standard curve of rat $ANP_{5\text{-}28}$ (atriopepin III). Results were normalized to cellular protein. Dexamethasone (10^{-7} *M*) doubled

the ANP release in our series (Klinger, unpublished data) and in another study, nearly tripled the ANP released from neonatal cardiomyocytes (18 pg/μg protein in controls versus 58 pg/μg protein after dexamethasone) (18).

Total RNA was extracted from the cultured neonatal rat cardiocytes using RNAzol B (CINNA/BIOTECX, Houston, TX). Cells were lysed directly in 6-well (33 mm/well) culture plates by the addition of 0.2 ml/well RNAzol B. The RNAzol B containing the cell extract was mixed with 0.1 volumes of chloroform, cooled to 4°C for 5 min, and then centrifuged at 12,000*g* for 15 min. The aqueous phase was removed and mixed with an equal volume of isopropanol, allowed to cool to −20°C overnight and centrifuged at 12,000*g* for 30 min the following day. The supernatant was removed and the RNA pellet was washed with 75% ethanol (0.8 ml/50–100 μg RNA) and centrifuged again at 7500*g* for 8 min to collect the RNA pellet. The RNA pellets were dried in a vacuum for 20 min and then were solubilized in DEPC treated ultra pure water containing 1 m*M* EDTA at pH 7. The mRNA was separated by electrophoresis on a 1.1% agarose gel and passively transferred to nitrocellulose membranes. Northern blots were hybridized with rat Type 1 11β-OHSD cDNA obtained from Dr. Perrin White (New York, NY) labeled by random priming (Boehringer Manheim, Indianapolis, IN) using ^{32}P dCTP (Amersham, Arlington, IL). This cDNA contains 1265 base pairs with an open reading frame of 861 base pairs (19). Following high-stringency washes, blots were exposed to XAR5 x-ray film (Kodak) with Lightening Plus (DuPont) intensifying screens for 4 hr to 6 days at −70°C. The blots were then stripped and reprobed with a 1128 bp PCR product specific for human β actin (Clontech Laboratories, Inc., Palo Alto, CA) as a control for the amount of RNA loaded per lane. The quantitation of the Northern blots was done using computerized scanning densitometry with a Microteck 600 ZS flat bed scanner connected to a Macintosh computer. The data were expressed as a ratio of the 11β-OHSD to the β actin signal and also 11β-OHSD to 18S ribosomal RNA from the same lane.

All results were expressed as the mean ± standard error. Data were analyzed for statistical purposes using the Student's *t* test with significance determined at $P < 0.05$. Data were normalized where appropriate to the protein content within individual samples. Proteins were determined using the Bradford protein assay (Bio-Rad, Richmond, CA). The mean protein concentration for the heart minces was 0.93 ± 0.11 mg/ml.

Results

The newborn rat heart demonstrated significant 11β-OHSD activity which did not appear to change over the first 8 days of life. Tissue minces obtained from animals sacrificed at 1 day of age generated 1.16 $\pm$ 0.19 $\times$ 10^{-12} moles of compound A per milligram of protein over a 60-min incubation period. By Day 8, litter mates generated 1.25 $\pm$ 0.35 $\times$ 10^{-12} moles of compound A per milligram of protein over the same time period.

According to protocol, rat pups either received the vehicle; corticosterone (the endogenous glucocorticoid for a rat) in doses of 20 μg/pup/injection, 200 μg/pup/injection or 1 mg/pup/injection; or dexamethasone 5 μg/pup/injection on Day 2–6 of life. As mentioned, hearts from 8-day-old control rat pups generated 1.25 $\pm$ 0.35 $\times$ 10^{-12} moles of compound A per milligrams of protein (10.3% ± 1.1% transformation) during the 60-min incubation with 10^{-8} *M* ^{3}H-corticosterone. Cardiac tissue derived from rats that were pretreated with corticosterone during Day 2–6 demonstrated a similar degree of 11β-OHSD activity when compared with controls 1.17 $\pm$ 0.14 $\times$ 10^{-12} moles compound A/mg protein (10.3% ± 1.2% transformation), medium B 1.19 $\pm$ 0.15 $\times$ 10^{-12} moles compound A/mg protein (9.9% ± 1.4% transformation), and high B 1.08 $\pm$ 0.17 $\times$ 10^{-12} moles compound A/mg protein (7.6% ± 1.0% transformation), respectively. In contrast, cardiac tissue derived from rat pups previously treated with dexamethasone demonstrated a marked decrease in 11β-OHSD activity assessed by a significantly lower amount of generated compound A, 3.18 $\times$ 10^{-13} moles compound A/mg protein (4.5% ± 1.0% transformation) ($P < 0.05$ when compared with moles generated in controls) (Fig. 1). Figure 2 is a representative HPLC illustrating the effect of dexamethasone on 11β-OHSD activity. Thus, prior exposure to dexamethasone decreased 11β-OHSD activity, while administration of comparable doses of endogenous glucocorticoid corticosterone failed to affect enzyme activity.

In order to determine whether prior dexamethasone treatment resulted in altered 11β-OHSD expression in cardiac myocytes, 11β-OHSD specific mRNA was quantified in cultured rat heart cells by Northern hybridization. Densitometric analyses of Northern blots were consistent with a reduced level of 11β-OHSD mRNA in cultured cardiomyocytes previously exposed to dexamethasone for 4 days (Table I).

Discussion

Our observation that cardiomyocytes derived from the rat pup exhibit 11β-OHSD activity contrasts with an earlier report by Funder and his colleagues (4), who were unable to demonstrate enzyme activity in hearts derived from week-old rats. Walker and his associates, however, were clearly able to show that 11β-OHSD was present and active in hearts derived from mature rats (7). Using immunohistochemical methods and a rabbit derived polyclonal antibody against hepatic 11β-OHSD, those investigators demonstrated

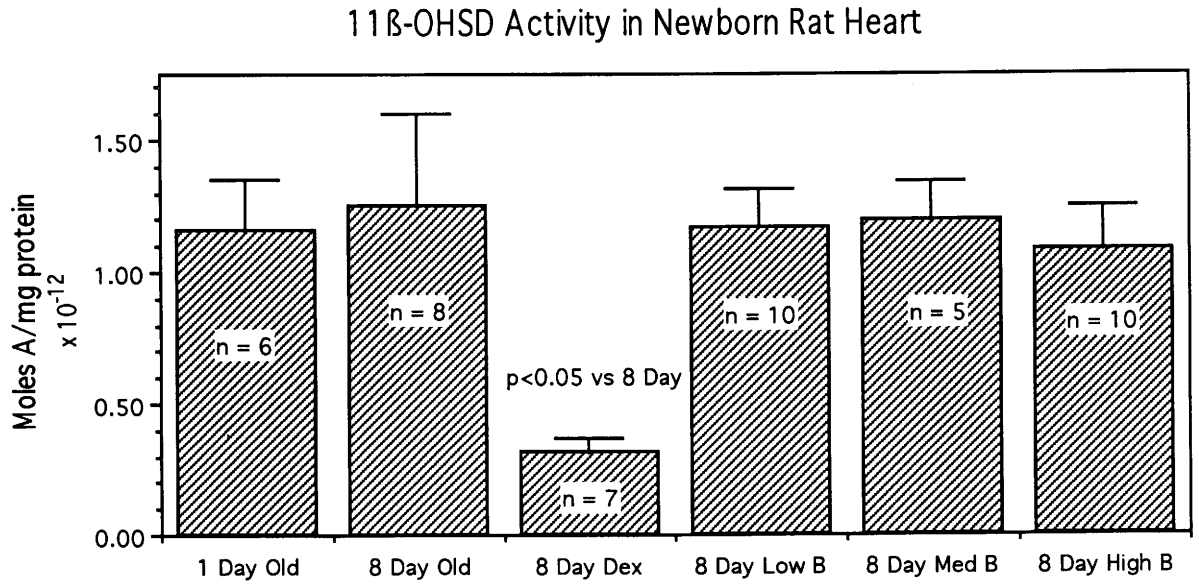

Figure 1. Effect of glucocorticoid pretreatment on 11β-OHSD activity in heart minces prepared from the rat heart. Animals were administered vehicle, corticosterone (20 or 200 μg/pup/injection, or 1 mg/pup/injection), or dexamethasone (5 μg/pup/injection) during the first week of life.

that the enzyme was located in cardiomyocytes; homogenates prepared from those cells were also able to convert corticosterone to its 11-dehydro derivative (7). Type I isoform of 11β-OHSD is the likely enzyme present in cardiac tissue based on the previously reported requirement for $NADP^+$ (20), the use of an antibody directed against the hepatic isoform of the enzyme (7), and the current findings with the type 1 cDNA. In the present studies and in those conducted by Walker, the concentration of corticosterone used to assess 11β-OHSD activity was relatively low at 10^{-8} *M*. The use of higher concentrations of labelled steroid substrate (corticosterone) may have masked detection of lower degrees of enzyme activity in the earlier experiments.

Cardiac myocytes derived from rat pups demonstrate a significant amount of 11β-OHSD activity which does not appear to change with active growth during the first 8 days of life. Interestingly, administration of pharmacologic doses of the endogenous glucocorticoid corticosterone did not influence either enzyme activity or the structural appearance of the heart during this crucial period of development. Excess corticosterone most probably is metabolized by preexisting enzymes both in the heart and in liver. 11β-OHSD activity appears to be low in the developing newborn kidney (21) but is clearly high in the newborn liver (22). In contrast to corticosterone treatment, animals given dexamethasone, a synthetic glucocorticoid, may have a limited ability to metabolize the excess steroid. Dexamethasone itself is a poor substrate for type I 11β-OHSD and generally thought to be metabolized to a limited extent by either the type II isoform of 11β-

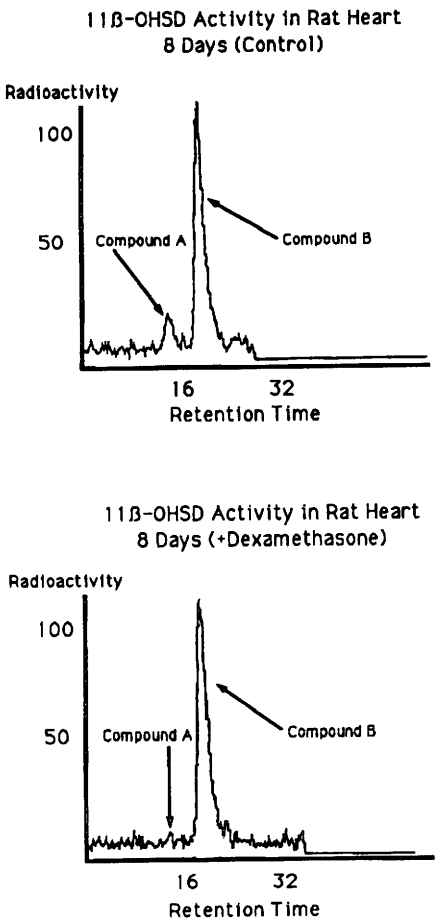

Figure 2. A representative HPLC illustrating 11β-OHSD activity (the conversion of corticosterone to 11-dehydrocorticosterone) in control hearts and in hearts from animals pretreated with dexamethasone.

Table I. Effect of Dexamethasone on the Expression of 11β-OHSD Isolated from Cultured Rat Cardiomyocytes Normalized to β Actin mRNA and 18s RNA

Conditions	11β-OHSD/ β Actin RNA	11β-OHSD/ 18s RNA
Cardiomyocyte (Control)	2.06	1.62
Cardiomyocyte (+ Dexamethasone 10^{-8} *M*)	0.77	0.67
Cardiomyocyte (+ Dexamethasone 10^{-7} *M*)	0.47	0.37

OHSD contained in renal cortical collecting tubules and possibly other sites and by the P450 6β-hydroxylation pathway (23, 24). The slow metabolism of this synthetic steroid is most likely related to the 9α-fluoro group in the B steroid ring. From our studies, the presence of dexamethasone is associated with a decrease in cardiac 11β-OHSD activity, a decrease in 11β-OHSD mRNA expression, and the structural abnormalities previously noted. In contrast, fibroblasts previously exposed to either corticosterone (25) or dexamethasone (26) in the culture medium show increased 11β-OHSD activity rather than the suppression we have observed in more mature cardiomyocytes. Whether the stage of cell differentiation accounts for this difference is unclear.

Short-term exposure to dexamethasone induces disseminated collagen deposition and reduced cell density in the newborn rat myocardium (27). These findings are similar to the myocardial fibrosis and hypertrophy associated with the chronic administration of mineralocorticoids including aldosterone and DOC (13, 14). The changes in the heart following mineralocorticoid exposure correlate with a net increase in the amount of tissue collagen and appear to be independent of the effects of mineralocorticoid-induced hypertension. The precise mechanisms involved with evolution of the cardiac structural changes in both these situations—specifically which hormones and receptors are involved—clearly remains to be elucidated. From our enzyme studies and Northern blot analyses, the cardiac hypertrophy observed with chronic administration of dexamethasone to newborn infants and the newborn rat pup correlates with down-regulation of 11β-OHSD synthesis and activity. Moreover, chronic dexamethasone treatment appears to enhance type I mineralocorticoid receptor expression without a corresponding change in the type II glucocorticoid receptor in cardiomyocytes (9). Thus, it is conceivable that chronic dexamethasone exposure alters receptor number and/or sensitivity to mineralocorticoids. By decreasing cardiac 11β-OHSD, dexamethasone could also allow for increased corticosterone binding to mineralocorticoid receptors. Generally, dexamethasone suppression of ACTH-dependent adrenal glucocorticoid secretion is incomplete, reducing endogenous glucocorticoids by about two thirds, and the effect of dexamethasone subsides within 24 to 48 hr after the last dose. Our observations raise the possibility that chronic treatment of neonates with dexamethasone may pose risks over and above those normally considered.

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